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*EFFECT OF RIBONUCLEASE ON CROSSING OVER IN DROSOPHILA**

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Communicated December 6, 1956

It has been demonstrated recently that the frequency of crossing over and genetic recombination can be increased significantly by exposure of plants and animals to agents or conditions capable of modifying the intracellular ionic environment.[1] The experimental evidence has been derived primarily from studies employing the sequestering agent, ethylenediamine tetraacetic acid (EDTA), which effects the chelation of a large number of metallic ions. In assessing the mode of action of EDTA in modifying crossing over, attention has been focused on the possibility that disruption of linear continuity of the chromatids incidental to recombination is facilitated by removal of Ca and Mg ions essential to maintenance of structural integrity. The alternative interpretation that some more general disturbance of the ionic environment might be the essential predisposing factor has not been overlooked[1] but has been discounted because of presumedly commanding evidence of the specificity of action of EDTA in chelating intrachromosomal divalent cations. This evidence rests on the observations that cells often contain large amounts of Ca and Mg,[2] that chromosomes break spontaneously with high frequency in plants growing in a medium deficient in these ions,[3] and that chromosomes (in tissues fixed by freezing) undergo marked deformation when exposed to the action of low concentrations of EDTA.[4]

With respect to the observations of the last category, evidence obtained in this laboratory indicates that the type of deformation effected by EDTA can be induced by chemical agents lacking specificity of action in removal of Ca and Mg ions. In such cases some general modification of the ionic environment of the cell appears to be involved, which so alters the properties of structural nucleoproteins that they are capable of undergoing enormous swelling or shrinking.[5] Correlated cytochemical and chemical analyses suggest that nucleic acids play a major role in determining the nature and extent of the concomitant cellular deformation. Other experiments indicate that EDTA has a marked, although probably indirect, effect on ribonucleic acid (RNA).[5] These findings served to cast a new light on the problem of the mode of action of EDTA on crossing over, and prompted the studies reported here, which were undertaken to determine whether ribonuclease as well as EDTA can modify recombination values in *Drosophila*.

Materials and Methods.—The white, miniature, forked (*w m f*), sex-linked genes of *D. melanogaster* were used to determine the effects of ribonuclease and EDTA on recombination frequencies. The ribonuclease was employed in a 1 mg/ml (0.00007 *M*) aqueous solution adjusted to pH 7 with 0.01 *M* NaOH, the EDTA in a 0.01 *M* aqueous solution of the same pH.[5] Early-third-instar female larvae (from pair matings between *w m f* and *Sw b* parents) were exposed to the action of these agents in either of two ways: by injecting about 0.3 ml. into the abdomen near the gonads or by immersing the larvae in the solution to be tested at 23° C. for about 8 hours. Effectiveness of the latter as well as of the former method was indicated by failure of a proportion of the larvae to pupate and of pupae to eclose. Since treatment by submersion was the less laborious experimental procedure, it was employed to obtain the bulk of the data presented in this report.

Individuals that had been treated as larvae by submersion in water adjusted to pH 7 or by injection with insect Ringer's solution served as one set of controls. Another set (designated as "untreated") consisted of individuals that had not been subjected to any experimental treatment. All cultures were maintained at 23° C. throughout the course of the experiments. Upon their emergence, the heterozygous females were mated individually in vials with *w m f* males. They were transferred thereafter to fresh food at 2-day intervals over a period of 16 days and were then discarded. The adults developing from the eggs laid during the eight 2-day periods afforded the data considered below.

Results.—The total progeny obtained from each of the four types of heterozygous females (ribonuclease-, EDTA-, and water-immersed, and untreated) are grouped in Table 1 with respect to noncrossover and crossover classes. A comparison of the four distributions by the χ^2 method reveals that the series is significantly heterogeneous and that the differences cannot be attributed to sampling errors ($P < .0001$).

TABLE 1

FREQUENCIES OF NONCROSSOVER AND CROSSOVER TYPES AMONG PROGENY OF ♀♀ HETEROZYGOUS FOR *w m f*, SUBJECTED AS LARVAE TO TREATMENT INDICATED

(Distribution Tested for Homogeneity by χ^2 Method)

	Non-crossovers	Single Crossovers, *w-m*	Single Crossovers, *m-f*	Double Crossovers, *w-m-f*	Total
Untreated	10,967	4,503	2,814	494	18,778
Treated by submersion:					
RNase*	13,168	5,854	3,731	676	23,429
EDTA†	15,861	6,657	4,202	710	27,430
Water‡	17,740	7,753	4,636	804	30,933
Total	57,736	24,767	15,383	2,684	100,570
Percent	(57.41)	(24.63)	(15.30)	(2.67)	(100.01)
	$\chi^2 = 32.74$;	$N = 9$;	$P < .0001$.		

* 0.00007 *M* solution, pH 7.0.
† 0.01 *M* solution, pH 7.0.
‡ At pH 7.0.

A more precise determination of the factors contributing to the heterogeneity is obtained by comparing the different distributions in all possible combinations of pairs and groups of three (Table 2). Such comparisons indicate that only the progeny of the ribonuclease-treated females differed significantly from all the others with respect to the pattern of distribution of crossover and noncrossover

types. This difference is not attributable to differential survival of complementary classes of offspring; comparison by the χ^2 method of the distributions of the four

TABLE 2

COMPARISON, BY PAIRS AND GROUPS OF THREE, OF DIFFERENT DISTRIBUTIONS OF NONCROSSOVER AND CROSSOVER TYPES LISTED IN TABLE 1 USING χ^2 TEST FOR HOMOGENEITY

	χ^2	N	P
Ribonuclease-untreated-water	28.74	6	$<.0001$
Ribonuclease-untreated-EDTA	25.61	6	*Ca.* .0003
Ribonuclease-EDTA-water	23.30	6	*Ca.* .0007
Ribonuclease-EDTA	15.46	3	*Ca.* .0015
Ribonuclease-untreated	21.46	3	$<.0001$
Ribonuclease-water	14.74	3	*Ca.* .002
EDTA-untreated-water	10.28	6	*Ca.* .12
EDTA-untreated	1.94	3	*Ca.* .6
EDTA-water	5.35	3	*Ca.* .15
Untreated-water	7.88	3	*Ca.* .05

pairs of complementary classes among the progeny of the four groups of heterozygous females reveals in each case a high degree of homogeneity, the four values of P falling between .2 and .55. Moreover, the pattern of crossing over is essentially similar in all four distributions, since the coefficients of coincidence range around .70 and the deviations therefrom are not statistically significant.

That the difference between the effects of the various kinds of treatment is due primarily to the high frequency of recombination induced by the action of ribonuclease is shown graphically in Figure 1. The curves given there indicate the per-

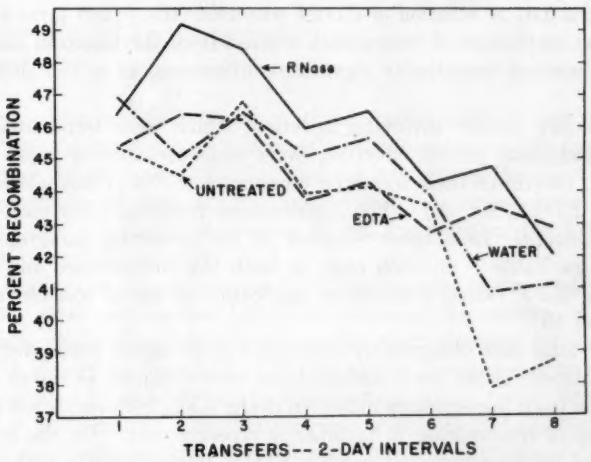

FIG. 1.—Percentage of recombination in the *w-m-f* region of the X chromosome of *D. melanogaster* for each of eight 2-day transfer periods. Crossover and noncrossover types detected by examination of adults developing from eggs laid by heterozygous females, which either were exposed as larvae to the agents indicated (EDTA, ribonuclease, or water) or remained untreated.

centage of recombination detected by examination of adults developing from eggs laid during each of the eight 2-day transfer periods. It is apparent that the ribo-

nuclease values exceed all others, except for the first and last transfers. Statistical analysis confirms the inference to be drawn from the graphic demonstration of Figure 1 that the progeny obtained from eggs laid during the period extending from the second to sixth days after mating made the major contribution to the high frequency of recombination detected in the offspring of ribonuclease-treated females.

The data assembled in Table 1 have also been considered with respect to the percentage of crossing over in each of the two regions tested, namely, *w-m* and *m-f*. Comparisons of the observed differences in terms of their standard errors indicate that the frequencies of recombination resulting from exposure of larvae to the action of ribonuclease are, with one exception (ribonuclease versus water for the *w-m* region), significantly higher than those caused by any of the other treatments. Considered quantitatively, the greatest effect of ribonuclease in increasing crossing over was detected in the *m-f* region, which thus appears to be particularly responsive to the action of this enzyme.

The distributions of crossover and noncrossover types reported above were obtained in experiments involving treatment of larvae by submersion. Comparable distributions were obtained when the larvae were injected with concentrations of ribonuclease or EDTA equivalent to those used in the immersion experiments. Thus the frequencies of crossover and noncrossover types were similar among the progeny of mothers exposed by injection and by immersion to the action of a 1 mg/ml solution of ribonuclease (the χ^2 test for homogeneity affords a value of P of .75); the frequencies obtained after injection and after immersion were also similar when a 0.01 M solution of EDTA was used (the χ^2 test gives a value of P of .85). The coefficients of coincidence derived from the injection data all range around .70, without statistically significant differences, as in the submersion experiments.

In preliminary studies involving injection, which were terminated when the immersion technique proved effective, other concentrations of these two agents were used to determine their action on crossing over (viz., ribonuclease, 0.01, 0.1, 0.2 mg/ml; EDTA, 0.1 M). The distributions resulting after such treatments differed significantly from those reported in the preceding paragraph and from those given in Table 1 (in each case, in both the ribonuclease and the EDTA comparisons, the P values obtained in application of the χ^2 test for homogeneity were less than .01).

When the total data obtained by injecting a given agent were grouped, regardless of the concentrations used, distributions were obtained as shown in Table 3. The deviation from homogeneity indicated in the table, however, is not attributable to the action of ribonuclease in facilitating crossing over. On the contrary, the percentage of crossing over induced by EDTA is significantly higher than that induced by either ribonuclease or Ringer's solution (the difference is about 2.5 times the standard error in each case). This difference reflects a high level of crossing over in the *w-m* region, which is attributable primarily to the action of the 0.1 M concentration of EDTA (compared with the ribonuclease data, the difference is 4.3 times the standard error). No significant difference between the effect of EDTA and that of ribonuclease or Ringer's solution was detected in the *m-f* region, although, as in the immersion experiments, the frequency of crossing over was greater in the ribonuclease-treated females than in the others.

All the data considered in the foregoing analyses were obtained by totaling the progeny of large numbers of treated females. The plan, as originally conceived, of scoring the progeny of individual mothers was designed to detect any possible influence of treatment of larval gonads on the chromosomes of oögonial cells. A few aberrant distributions were detected among the progeny of the ribonuclease- and EDTA-treated females, which suggested that oögonial crossing over might have preceded meiotic crossing over,[6] but comparable distributions were also observed among the progeny of the untreated and water-immersed larvae. Small numbers are necessarily involved when the progeny of individual females are considered, and statistical evaluation of the data indicated that the variability could be attributed to sampling errors. For this reason the data were grouped to give the distributions presented in Tables 1–3 of this report.

TABLE 3

FREQUENCIES OF NONCROSSOVER AND CROSSOVER TYPES AMONG PROGENY OF ♀ ♀, HETEROZYGOUS FOR *w m f*, INJECTED AS LARVAE WITH AGENTS INDICATED

(Distribution Tested for Homogeneity by χ^2 Method)

	Non-crossovers	Single Crossovers, *w-m*	Single Crossovers, *m-f*	Double Crossovers, *w-m-f*	*Total*
RNase*	1,732	673	503	73	2,981
EDTA†	2,739	1,262	779	140	4,920
Ringer's‡	491	186	124	19	820
Total	4,962	2,121	1,406	232	8,721
Per cent	(56.90)	(24.32)	(16.12)	(2.66)	(100.00)

$\chi^2 = 14.61$; $N = 6$; $P = ca.\ .025$.

* Combined data for 1.0, 0.2, 0.1, and 0.01 mg/ml, pH 7.
† Combined data for 0.01 and 0.1 *M* solutions, pH 7.
‡ Insect Ringer's solution.

Discussion.—The results obtained in these studies suggest that the processes involved in meiotic crossing over and genetic recombination can be modified by the action of either ribonuclease or EDTA, that the intensity of the effect is a function of the concentration of each of these agents, and that ribonuclease tends to act preferentially in increasing recombination within one region of the X chromosome, EDTA within an adjoining region.

The ability of either ribonuclease or EDTA to increase the over-all frequency of recombination in *D. melanogaster* brings into question the validity of assumptions about the dependence of such an increase on the direct chemical action of a specific agent. With respect to the effect of EDTA at the chromosomal level, the possibility of removal of Ca and Mg ions by chelation cannot be denied, but the further inference that this reaction serves to uncouple gene loci and facilitates recombination is subject to debate. As noted elsewhere, the chromosomes of frozen-thawed cells and of unfixed cells respond to the action of EDTA by swelling and undergoing marked deformation, but they do not fragment.[5]

More specifically, the findings of the present study (and also those of Levine,[1] which were derived from analysis of the progeny of females that had been fed EDTA during the third instar of larval life) lend little support to the premise that augmentation of crossing over in *Drosophila* depends on the direct chelation of divalent cations within the meiotic chromosomes. Gonads of early-third-instar larvae contain only oögonia,[7] which, as noted above, are not markedly affected by

the treatment. The oöcytes do not appear until shortly before the emergence of the fly.[7] Effects on meiotic crossing over in types of cells that had not eventuated at the time of exposure of the larva to the predisposing agent can best be assessed in terms of changes in the general ionic environment of the organism. This situation is not unexpected, in the light of earlier findings of the prolonged sensitizing action of near-infrared radiation in modifying the intracellular environment so as to influence the subsequent production of X-ray- and chemical-induced chromosomal interchanges.[8]

Apart from such objection to inferences about direct action, it may be noted that the increase in frequency of recombination resulting from treatment with EDTA was at best around 10–20 per cent (values that correspond closely with those obtained in other studies on *Drosophila*[1]). There is abundant evidence that modification of crossing over can be effected to an equal or greater extent by altering the genetic constitution or physiologic properties of the cell through application of a variety of experimental procedures.[9] It is difficult to equate these comparable effects of divers physical and chemical agents on crossing over in terms of the disruption of linear continuity of the chromosomes through removal of a ubiquitous bonding material.

Any assumptions about a direct relationship between the specificity of action of ribonuclease in degrading RNA and the facilitation of recombination are also subject to debate. As indicated elsewhere,[10] the action of ribonuclease on the living cell causes chromosomal adhesion and clumping through induced "stickiness" and impairment of the spindle mechanism, but the pathways involved remain largely unknown. One difficulty in establishing a direct correlation arises from the fact that many other agents having no known specificity of action on chromosomal materials can simulate ribonuclease in the production of mitotic abnormalities.[10]

Of special significance to the present discussion is the observation that ribonuclease was highly effective in increasing recombination within the *m-f* region, which extends from 36.1 to 56.7 on the genetic map and encompasses the intercalary heterochromatic regions lying in subdivisions 11*A* and 12*D, E*, of the salivary-gland chromosome. Cytochemical studies suggest that heterochromatic regions contain RNA,[11] but whether this nucleic acid is primarily involved in the ribonuclease-induced modification of crossing over would have to be determined by analysis of genetic recombination within much shorter regions of the X chromosome than are considered in this study. A relatively high coefficient of crossing over has been reported for heterochromatin when removed from the interference exerted by the centromere;[12] the further possibility that changes in the physicochemical properties of heterochromatic materials may be factors predisposing to enhanced recombination is suggested by the findings reported here.

These experimental results also focus attention on the necessity for critical examination of concepts of mechanisms of crossing over in terms of the chemical constitution of chromosomal materials and the changes effected therein during the meiotic prophases. This necessity is further emphasized by the observation that each chromatid is in itself polynemic or multistranded,[13] although functioning as an integrated unit in crossing over and genetic recombination.

Summary.—Results obtained in studies of the effects of ribonuclease and EDTA on crossing over in *D. melanogaster*, using the *w m f* sex-linked genes, indicate that

the frequency of recombination can be modified by either of these agents, that the intensity of the effect is a function of the concentration used, and that ribonuclease tends to act preferentially in increasing recombination within the *m-f* region, EDTA within the *w-m* region. The bulk of the data were obtained by examination of the progeny of heterozygous females that had been treated as third-instar larvae (when the gonads contain only oögonia) by submersion in either ribonuclease, EDTA, or water, or had remained untreated. Statistical evaluation of these data reveals that only the progeny of the ribonuclease-treated females differed significantly from all the others with respect to pattern of distribution of noncrossover and crossover types. In preliminary studies involving treatment by injection, using a higher concentration of EDTA than in the submersion experiments, the effect of EDTA was significantly greater than that of ribonuclease.

The findings suggest that neither ribonuclease nor EDTA modifies the process of crossing over and recombination solely by direct action on the chromosomes but that each causes changes in the nucleoprotein fabric as a consequence of alterations in the environment of the cell. The implications of the findings are discussed briefly.

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*THE NATURE OF THE CHANGES EFFECTED IN CHROMOSOMAL MATERIALS BY THE CHELATING AGENT EDTA**

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INTRODUCTION

Ethylenediaminetetra-acetic acid (EDTA) can cause deformation of chromosomes in plant and animal cells. This effect has been attributed to removal by chelation of divalent cations essential for maintenance of chromosomal integrity.[1] The underlying assumption that Ca and Mg ions serve to bond the macromolecules of chromosomal nucleic acids and proteins has been invoked in turn to explain the modifying action of chelating agents on crossing over and recombination.[2] Because of the genetic implications,[3] it seemed desirable to re-examine the cytological foundations on which these interpretations rest in the light of additional observational and experimental evidence. This report presents such evidence.

Although the findings sustain the view that EDTA can effect marked changes in the appearance and distribution of cellular materials, it does not necessarily follow that deformation of the chromosomal fabric depends exclusively on specific removal of its Ca and Mg ions. The alternative possibility should be evaluated that degradation results from changes in the colloidal properties of chromosomal materials as a result of more general alterations in the ionic environment of the cell. A pattern for this alternative was revealed in studies of the action of trypsin and pepsin on fixed cellular materials.[4] Each of these enzymes effected structural deformation, but not primarily through loss of protein following disruption of peptide linkages, since hydrolysis could be continued for long periods of time under appropriate experimental conditions without appreciable cellular distortion. Treatment with these proteases did so modify the properties of structural nucleoproteins, however, that they were capable of swelling or shrinking enormously when afforded a suitable ionic environment. Changes in nucleoprotein gels thus appeared to be the factor most immediately responsible for protease-induced deformation of cellular structures.

Experimental assessment of the alternatives indicated above with respect to the mode of action of EDTA provided evidence that this chelating agent can also cause chromosomal deformation by modification of the properties of constituent nucleoprotein gels. The findings are summarized below.

MATERIALS AND METHODS

Salivary-gland cells of larvae of *Drosophila melanogaster* and meristematic cells of roots of the onion (*Allium cepa*) were used in analysis of the mode of action of EDTA.

Salivary Glands.—The glands were dissected from the larvae while immersed either in Ringer's solution (Bodenstein;[5] ionic strength = 0.138, pH = 6.4) or in water at pH 6 or 7 and were frozen immediately in the same medium, using CO_2 as specified by Mazia.[1] (In a few supplementary experiments the glands were

dissected in Ringer's solution and transferred for freezing to EDTA or to the control solution that was to be tested.) After thawing, the glands were exposed for 2, 4, 6, 24, or 48 hours to the action of EDTA or control solutions at refrigerator temperature (*ca.* 4° C.), with occasional transfer to room temperature (*ca.* 22° C.) for purposes of microscopic examination. In those experiments in which treatment with enzymes preceded treatment with EDTA, the enzymatic hydrolysis was carried out at 37° C. in accordance with standard procedures developed in our earlier studies.[6]

Effects of the treatments were determined cytologically and cytochemically, using squashes or imbedded and sectioned material, or intact glands placed in a chamber[4] that permitted rapid and complete exchange of fluids and facilitated microscopic examination of the preparation during the course of treatment.

Solutions of EDTA were also tested on nonfrozen glands, either immediately after dissection or after fixation in 45 per cent acetic acid.

Onion Root Tips.—The tips, which had attained a length of 3–4 mm., were detached from bulbs sprouted in sand and used for chemical analysis. The detached tips were frozen immediately in water, homogenized at 0°–3° C. in the same medium, and stored at 3° C. in different concentrations of EDTA at pH 7. Aliquots were removed at various times and analyzed by a modification[7] of Schneider's procedure for acid-soluble constituents, phospholipids, nucleic acids, and proteins.

The EDTA solutions were prepared from an analytical reagent grade of disodium ethylenediaminetetra-acetate purchased from the Fisher Scientific Company. The desired pH's were obtained by titrating 0.2 *M* Na_2EDTA with 1 *M* NaOH and adjusting the final concentration to 0.1 *M*. Lower concentrations were prepared by diluting the 0.1 *M* stock solutions with water.

RESULTS

Deformation of Salivary-Gland Chromosomes.—The extent of degradational action effected by EDTA was found to depend on the medium in which the glands were frozen, the concentration of the chelating agent, and the duration of the treatment. In general, glands that had been dissected from the larvae in water at pH 6 and frozen in that medium revealed upon thawing and staining more chromosomal distortion (largely through vacuolization) than those frozen in water at pH 7, whereas the latter, in turn, showed less precise banding of chromosomes than the specimens frozen in Ringer's solution. The material frozen in Ringer's solution was therefore used to supplement the water-frozen material in assaying the effects of EDTA on chromosomes. Despite the mildness, in a chemical sense, of fixation by freezing in water or Ringer's solution, all preparations revealed, upon thawing, marked deviations—in cellular dimensions, contours of the nucleus, and form of the chromosomes—from the pattern disclosed in cells of glands immediately after their removal from the larvae in Ringer's solution. These unfrozen (unfixed) glands were very sensitive to the action of EDTA, as evidenced by dissociation of the cells and deformation of the contents. Glands fixed in 45 per cent acetic acid, on the other hand, appeared to be unaltered by long exposure to the action of EDTA.

Dissociation of cells was one of the more general effects of the action of EDTA on the frozen-thawed glands. The lower concentrations tested (0.001 and 0.01

M) also made the glands very sticky. The stickiness was at times so pronounced that the glands adhered to the walls of the treatment chamber or to the sides of the silicone-coated medicine droppers that were used to transfer the tissues from one container to another.

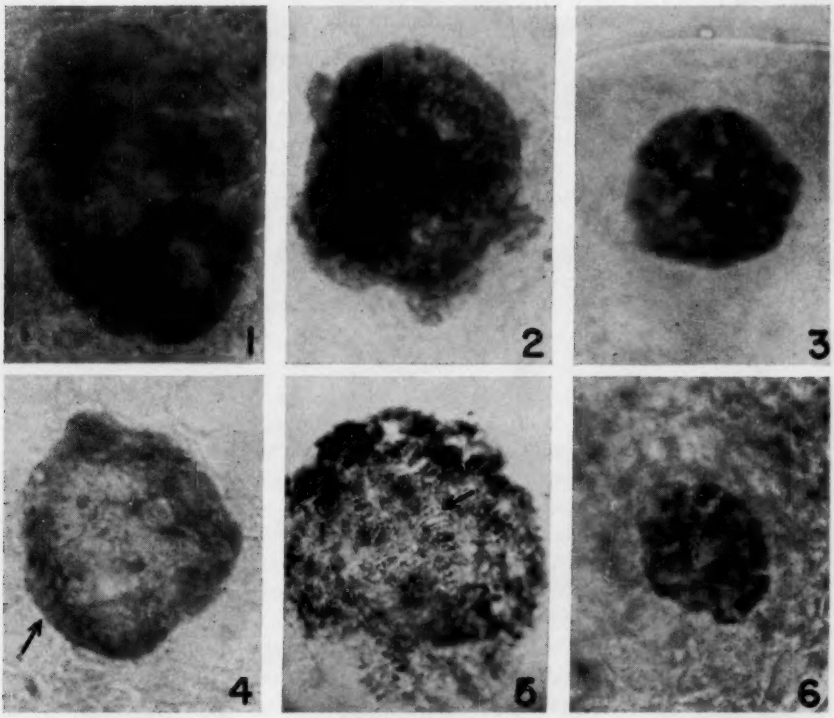
With respect to specific effects on chromosomes, 0.1 *M* EDTA when used for 2–6 hours preserved these bodies with a high degree of fidelity (Fig. 7), a 0.01 *M* solution gave some distortion (Fig. 12), and a 0.001 *M* solution produced a considerable amount of deformation (Fig. 1). These results are in essential agreement with those reported by Mazia.[1] In contrast with his findings, treatment with water alone either at pH 6 or pH 7 effected structural deformation in our material, which was somewhat less marked but differed only in degree from that produced by 0.001 *M* EDTA. After water treatment the glands were extremely soft and frangible, so that the chromosomes tended to disperse when the cells were squashed in acetic-orcein.

Cytological examination of orcein-stained whole glands or squashes after treatment with 0.001 *M* EDTA (Fig. 1) revealed that the nucleoli were swollen enormously and the chromosomes were displaced toward the nuclear membrane (cf. Fig. 4). The weak stainability of the chromosomes tended to obscure details of banding and to create the impression that structural continuity had been impaired. By immersing such treated material in acetic acid–alcohol for hardening, then sectioning and staining, it was, however, possible to determine that the chromosomes were present in a highly swollen state and had not undergone fragmentation.

Some additional lines of evidence support the interpretation that the action of EDTA does not lead to dissolution of the salivary-gland chromosomes. If cells that had been treated for 6 hours with a 0.001 *M* or a 0.01 *M* solution were rinsed thoroughly in water (at the same pH as that of the EDTA treatment), the chromosomes were usually more clearly defined than in the unrinsed controls (compare Fig. 2 with Fig. 1). When treatment with 0.001 *M* EDTA was prolonged for 24–48 hours, the pattern of banding of the chromosomes was often more precise than in glands treated for 6 hours (compare Fig. 3 with Fig. 1). The same type of response was detected in cells of glands that had been transferred to 0.1 *M* EDTA after treatment in a 0.001 *M* solution. As the cells shrank, it was observed that the highly dispersed materials of the banded regions tended to adhere to adjoining chromosomes with which they had established contact during the swollen state. The resulting interchromosomal connections created the appearance of a reticulate nucleus, but cytological examination of both squashes and sectioned material showed that the continuity of the individual chromosomes had not been impaired by fragmentation (Fig. 5).

In other experiments the glands were treated for 6 hours with 0.1 *M* EDTA and then rinsed thoroughly in water, a method regarded by Mazia[1] as equivalent to treatment with a lower concentration. Replacement of EDTA by water caused marked swelling of the nucleus, up to 40 times the original volume in some cases. The individual chromosomes then appeared vesiculate, with the "bands" dispersed laterally into component "chromomeres" (compare Fig. 7 with Figs. 8 and 9). Replacement of the water by 0.1 *M* EDTA again caused shrinkage. Each repetition of the swelling-and-shrinking process resulted in some loss of material and progressive deformation of the chromosomes. In these cases, also, microscopic exam-

ination of fixed and stained specimens afforded no evidence of disaggregation or breakage of chromosomal strands. A significant observation, with respect to evaluation of the mechanisms involved, is that neither 0.001 *M* nor 0.002 *M* $CaCl_2$ or $MgCl_2$, when used separately or in combination in place of water, exerted marked



FIGS. 1–12.—Effect of EDTA on salivary-gland cells of third-instar larvae of *Drosophila melanogaster*. All photographs are of "squash" preparations, but amount of squashing was determined by weight of cover slip. Methods of treatment are described in the text. Time of treatment: with EDTA, *ca.* 6 hours unless stated otherwise; with ribonuclease, *ca.* 2 hours. Figs. 1–6, 9–12, stained with acetic-orcein; Figs. 7 and 8, stained by Feulgen procedure. Note absence of chromosomal fragmentation in all figures and lateral dispersal of "bands" into "chromomeres," particularly in Figs. 4, 5, and 8–11, as indicated by arrows.

FIG. 1.—0.001 *M* EDTA.

FIG. 2.—0.001 *M* EDTA, then thorough rinsing in water.

FIG. 3.—0.001 *M* EDTA for 24 hours.

FIG. 4.—Same treatment as preceding; median optical focus shows swollen nucleolus as light-colored central area and peripheral displacement of chromosomes.

FIG. 5.—0.001 *M* EDTA, then 0.1 *M* EDTA.

FIG. 6.—Ribonuclease, 1 mg/ml, then water rinse, then 0.001 *M* EDTA.

inhibitory action on the swelling of the 0.1 *M* EDTA-treated cells (Fig. 10). In these experiments also the swelling was reduced by replacement with 0.1 *M* EDTA and induced again by use of the solution of divalent cations.

In an effort to determine whether EDTA-induced swelling involved nucleoproteins, glands were hydrolyzed with deoxyribonuclease, ribonuclease, or trypsin preparatory to treatment with EDTA. The action of deoxyribonuclease (0.125

mg/ml H_2O, pH 7, for 2 hours at 37° C.) was in itself so drastic, however, that little stainable chromosomal material was discernible for estimation of the subsequent action of EDTA. On the other hand, solutions of ribonuclease (1.0 mg/ml H_2O, pH 7, for 2 hours at 37° C.) induced only moderate structural deformation, including some swelling of nucleoli, without effacing the banded appearance of the chromosomes. It was determined cytochemically that these solutions were effec-

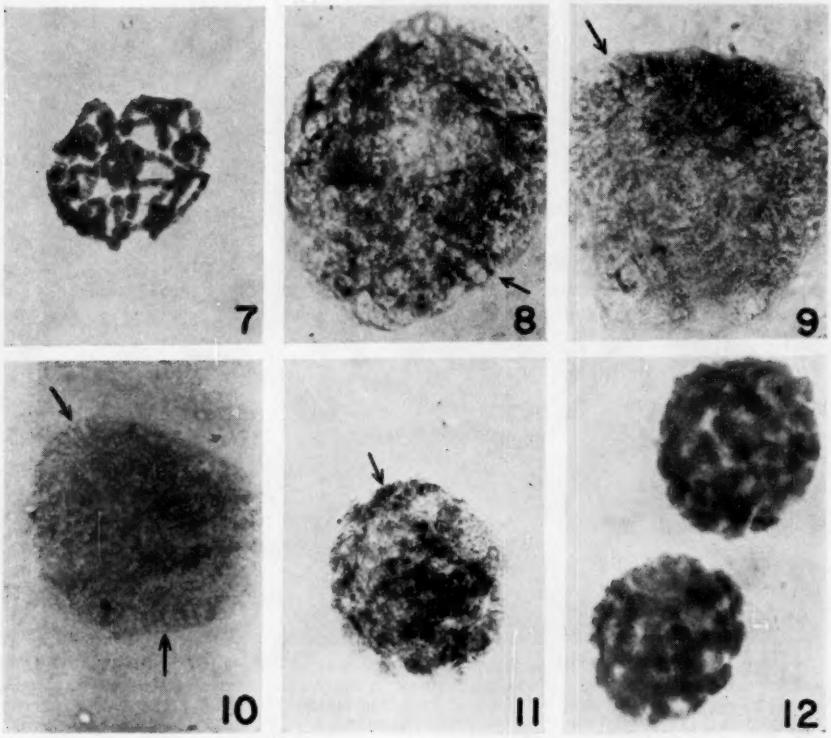


FIG. 7.—0.1 *M* EDTA.
FIG. 8.—0.1 *M* EDTA, then thorough rinsing in water.
FIG. 9.—Same as preceding, except for method of staining.
FIG. 10.—0.1 *M* EDTA, then 0.001 *M* $CaCl_2$.
FIG. 11.—Ribonuclease, 1 mg/ml, then 0.1 *M* EDTA, then water.
FIG. 12.—0.01 *M* EDTA.

tive in degrading ribonucleic acid (RNA). When such ribonuclease-hydrolyzed preparations were treated, after thorough rinsing in water, with 0.001 *M* EDTA, some additional swelling of nucleoli and lateral dispersion of the chromosomes occurred (Fig. 6), but the extent of deformation was much less than that in the controls, which had been kept in water at pH 7 for 2 hours at 37° C. before exposure to the action of 0.001 *M* EDTA. Similarly, glands exposed to a sequence of reagents, including ribonuclease, 0.1 *M* EDTA, and water, swelled only slightly (Fig.

11), whereas the controls, exposed under comparable experimental conditions to the sequence water, 0.1 *M* EDTA, and water, swelled enormously. Trypsin (0.1 mg/ml H_2O, pH 7, for 2 hours at 37° C.) in itself exerted marked deformational action on cells of glands that had been frozen in water, much less on cells of glands frozen in Ringer's solution. Subsequent treatment with 0.001 *M* EDTA increased the amount of distortion, but the vesiculate chromosomes revealed traces of banding and thereby furnished evidence that deformation had not been effected by disaggregation of the component chromonemata.

It appears from these experiments that RNA is involved in the deformational action of EDTA, since removal of this nucleic acid inhibited swelling. To determine whether EDTA per se has an effect on RNA, the EDTA-treated cells were stained with basic dyes that are used to identify this nucleic acid. It was then found that 0.001 *M* EDTA reduced basophilia markedly, that 0.01 *M* solutions gave slight reduction, and that 0.1 *M* solutions caused no appreciable reduction. Coincidentally with the decrease in stainability with basic dyes there occurred a marked increase in stainability with acidic dyes, suggesting that the action of 0.001 *M* EDTA had indeed served to uncover stainable groups on the proteins, presumably by degradation of the nucleoprotein complex.[8]

Solutions that were used as controls also effected cytologically discernible structural alterations. Sodium citrate (0.05 *M*), when used for 6 hours at pH 7, caused marked shrinkage of the glands. When stained, the margins of the nuclei appeared crimped, and the tightly aggregated chromosomes showed clearly defined bands. Removal or dilution of the citrate by water effected some swelling and structural deformation. Other solutions, containing 0.1 *M* $CaCl_2$ or $MgCl_2$ in 0.1 *M* EDTA, caused shrinkage of the nuclei and the aggregation of the chromosomes, to form tight little knots that were connected to the periphery by delicate chromatic strands. On the other hand, solutions containing 0.001 *M* $CaCl_2$ or $MgCl_2$ in 0.001 *M* EDTA effected nucleolar swelling and lateral dispersion of chromosomal materials. When the glands were rinsed in water after such treatment, there was marked additional swelling, so that, upon staining, the cells revealed large vacuolar nucleoli with peripherally disposed swollen chromosomes. In these preparations the chromocentral heterochromatic regions alone seemed to withstand the degradational action, standing out as compact, more densely staining islets among the loosely dispersed chromosomal materials.

Degradation of Onion-Root-Tip Constituents.—The essential experimental data on the effect of EDTA in degradation of various components of homogenates of frozen onion-root tips are summarized in Table 1. It is evident that the spontaneous autolysis of protein was slight and was not altered by the action of EDTA. This chelating agent, however, in concentrations greater than 0.001 *M* did inhibit the degradation of phospholipid and of deoxyribonucleic acid (DNA). The spontaneous autolysis of RNA, on the other hand, was markedly accelerated by 0.01 *M* EDTA, less so by 0.001 *M*, and not at all by 0.1 *M*. It should be noted that the degradational changes occurring in these phosphorus-containing components were reflected in the acid-soluble constituents, as indicated in Table 1. It is evident from these considerations that the most pronounced effect of EDTA under the experimental conditions specified was on the RNA component of the root-tip cells.

TABLE 1

EFFECT OF EDTA ON THE SPONTANEOUS DEGRADATION OF VARIOUS CONSTITUENTS OF ONION-ROOT-TIP HOMOGENATES AT 3° C., pH 7

EDTA (mM/l)	TIME (HOURS)	CONSTITUENT MEASURED (μg P/ml*)				
		Protein	Acid-soluble Phosphorus	Phospho-lipid	DNA	RNA
0	0	102	35.8	15.1	12.5	31.1
0	18	98.4	49.0	10.9	9.92	27.0
0	50	90.7	58.6	7.27	7.38	24.9
1	0	105	38.7	15.1	12.4	31.7
1	18	99.0	56.7	9.12	9.72	22.1
1	50	93.6	62.9	7.55	9.83	17.7
10	0	108	38.5	15.4	12.0	31.1
10	18	98.1	63.7	12.2	11.3	10.1
10	50	95.1	70.4	9.30	11.5	7.82
100	0	45.8†	37.5	15.7	12.3	31.2
100	18	44.6	43.4	14.2	11.9	28.1
100	50	43.0	50.1	11.4	12.4	24.4

* With the exception of the protein values, which are expressed in arbitrary units (see n. 7).
† These values were consistently low, owing to interaction between the EDTA and the CCl_3COOH in the fractionation process.

DISCUSSION

The results reported above focus attention on a pattern of response of frozen-thawed cellular materials to EDTA that involves alteration in the gel-like properties of structural nucleoproteins. Such alteration is revealed in a pronounced stickiness of the treated cells (a type of response that can be induced in chromosomes of living cells by a variety of physical and chemical agents, including ionizing radiations and ribonuclease[9]), in swelling of nucleoli and peripheral displacement of chromosomes, and in lateral distension of the chromosomal fabric, with separation of the "bands" into component "chromomeres."

That these effects depend on modification of the general ionic environment of the cell and not merely on the selective removal of specific divalent cations from the fabric of the chromosome is evidenced by a series of findings. Water, in itself, exerts some degradational action on frozen-thawed chromosomes; the extent of cytologically detectable deformation effected by low concentrations of EDTA can be reduced by prolonging the treatment, by thoroughly rinsing the cells in water, or by immersing them in a higher concentration of the chelating agent.[10]

Evidence that nucleoproteins are involved in structural deformation of chromosomes is afforded by the fact that pretreatment with ribonuclease inhibits swelling and by the nature of the changes produced in substrate materials. The experimental results suggest that RNA is implicated in the EDTA-induced response. The sensitivity of this nucleic acid to the action of EDTA is further emphasized by the chemical analysis of root-tip homogenates. The results do not necessarily denote a direct action of EDTA on RNA-containing substrates, since, alternatively, an effect on the cellular enzymes concerned with autolysis might be involved. It is evident that, in any enzyme system affected by inorganic ions, chelation will produce either an elevation or a depression of the observed enzymatic activity, depending on the relative availability of activating or inhibiting ions. For example, degradation of RNA by bovine pancreatic or pea-leaf ribonuclease is markedly dependent on the ionic composition of the digestion medium.[11]

The findings of the present study thus correspond in their broad, general implications with those derived from analysis of the action of proteolytic enzymes. Both studies reveal that changes had occurred in the gel-like properties of nucleoproteins

incident to alteration in the ionic environment of the cell. In one case this was attributable to the action of proteases of foreign origin, in the other case to the experimental "activation" of nucleases of endogenous origin (although it should be noted that the acetic acid–alcohol fixation that was conducive to protease-induced swelling appeared to inhibit completely any degradation by EDTA). Both types of treatment (with trypsin or EDTA) produced structural deformation and loss of chromosomal integrity through swelling and softening of essential structural components and not by direct disruption of linkages essential to maintenance of linear continuity. Both sets of results point to the conclusion that any agent capable of influencing the nucleoproetin constituents of the cell, either by modification of the ionic environment or by removal of some essential cellular materials, can lead to deformational changes in the chromosomes.[12]

The present report thus stands at variance with some of the earlier interpretations of the mode of action of chelating agents in effecting the degradation of chromosomes. In the light of this alternative point of view, it becomes necessary to reassess those theories of chromosome structure that have emerged from assumptions about the specific action of EDTA, and to find more substantial experimental evidence than has heretofore been presented to support the interpretation that bonding by divalent cations is essential to maintenance of chromosomal integrity. With respect to the inferences derived from studies of modification of crossing over by the action of EDTA on the chromosomes of living auxocytes, it might be noted that experiments conducted in this laboratory indicate that RNA may also be involved, so that the focus is again brought to bear on the structural nucleoproteins, as will be reported in detail in another publication.

SUMMARY

Evidence is presented that the deformational changes effected in plant and animal chromosomes by EDTA are referable to alterations in the colloidal properties of structural nucleoproteins. Cytological studies of salivary-gland cells of *D. melanogaster* indicated that 0.001 *M* EDTA causes marked swelling of nucleoli, with peripheral displacement of the laterally dispersed chromosomes. No evidence was obtained that 0.1, 0.01, or 0.001 *M* EDTA impairs the structural continuity of chromosomes by effecting their breakage or fragmentation. Cytochemical studies of salivary-gland cells and chemical analyses of onion-root-tip cells indicated that RNA is involved in the deformational changes. The findings lead to the conclusion that EDTA-induced chromosomal changes depend on modification of the general ionic environment of the cell and not merely on the selective removal of specific divalent cations from the fabric of the chromosomes. The implications of the findings with respect to theories of chromosome structure are discussed briefly.

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THE ASYMPTOTIC DISTRIBUTION OF EIGENFUNCTIONS AND EIGENVALUES FOR SEMI-ELLIPTIC DIFFERENTIAL OPERATORS

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The asymptotic distribution of eigenvalues and eigenfunctions of boundary-value problems for an elliptic partial differential operator has been intensively investigated in recent years, using the Tauberian argument introduced by Carleman.[1] Carrying out a detailed estimation of the fundamental solution in the small for $(A + t)$ obtained by the parametrix method, Gårding has obtained the principal term in the asymptotic behavior of the spectral function of a self-adjoint semibounded realization of an elliptic A,[2] obtaining also the eigenvalue distribution in the case of the Dirichlet problem.[3] The author has published a proof of the asymptotic distribution of eigenvalues and eigenfunctions for the Dirichlet problem[4] based upon a simpler estimation procedure using energy integrals, similar to one that had been earlier applied by Gårding in the case of constant coefficients.[5] It is the purpose of this note to show how that method may be applied to give asymptotic estimates for self-adjoint semibounded realizations of a semi-elliptic operator.

The eigenvalue distribution for variational boundary-value problems is obtained for smooth domains with both self-adjoint and non-self-adjoint operators. In a detailed exposition we shall give an extension of this method to the case of complete operators of local type with variable coefficients.[6]

Let G be an open set in the Euclidean space E^n of $x = (x_1, \ldots, x_n)$, R^n the dual space of $\xi = (\xi_1, \ldots, \xi_n)$ with $\langle x, \xi \rangle = \sum x_j \xi_j$. If α is the multi-index $(\alpha_1, \ldots, \alpha_k)$, m the n-vector $(m_1, \ldots, m_n)$ with positive integer components, $|\alpha| = k$, $|\alpha|_m = \sum_{j=1}^{k} m_{\alpha_j}$, $D_j = (1/i)\partial/\partial x_j$, $D^\alpha = D_{\alpha_1} \ldots D_{\alpha_k}$, $\xi^\alpha = \xi_{\alpha_1} \ldots \xi_{\alpha_k}$, $\xi_m{}^\alpha = (\xi_{\alpha_1})^{m_{\alpha_1}} \ldots (\xi_{\alpha_k})^{m_{\alpha_k}}$. Let $(,)$ be the inner product in $L^2(G)$, $C_c^\infty(G)$ the family of infinitely differentiable functions with compact support in G. If $A = \sum a_\alpha(x) D^\alpha$ is a partial differential operator with coefficients in $C^\infty(G)$, A is formally self-adjoint if $(A\phi, \psi) = (\phi, A\psi)$ for $\phi, \psi \in C_c^\infty(G)$. We shall say that A is semi-elliptic at x_0 with respect to the $(n+1)$-vector $(m_1, \ldots, m_n, q)$ of positive integers if there exists a neighborhood N of x_0 and a C^∞-co-ordinate system on $\bar{N}$, $\{y_j\}$, such that: (1) $A = \sum_{|\alpha|_m \leq q} a_\alpha'(y) D^\alpha{}_y$ for y in N; (2) if $A_0(y, \xi) = \sum_{|\alpha|_m = q} a_\alpha'(y) \xi^\alpha$, then $A_0(y, \xi_m)$ is a positive-definite q-form in ξ. If $2s_j = q(m_j)^{-1}$, s_j is a positive integer, and, for suitable c, $C > 0$, $C\sum_j \xi_j^{2s_j} \geq A_0(y, \xi) \geq c\sum_j \xi_j^{2s_j}$. Applying a Fourier transform argument, if we set $\|\phi\|^2_{H_p(N)} = \int_{R^n} A_0(x, \xi)^p |\hat{\phi}(\xi)|^2 \, d\xi$, $\hat{\phi}(\xi) = F(\phi)(\xi) = (2\pi)^{-n/2} \int \exp(-i\langle x, \xi \rangle) \phi(x) dx$ for $\phi \in C_c^\infty(N)$, we obtain

$$C_p \sum_j \|D_j^{s_j}\phi\|^2 \geq \|\phi\|^2_{H_p(N)} \geq c_p'(A^p\phi, \phi) + t_p(\phi, \phi) \geq c_p'' \sum \|D_j^{s_j}\phi\|^2.$$

It follows that if a distribution ζ_0 is a solution of the equation $(A^p + t)\zeta_0 = u$, where $u = \sum D_j^{s_j} f_j$ with $f_j \in L^2(N)$, then $D_j^{2ps_j - k_j}\zeta_0$ are locally square-summable. Since δ_x is the $[n/2]+1$ derivative of a locally square-summable function, if $g_{x,t}{}^{(p)}$ is a solution of $(A^p + t)g_{x,t}{}^{(p)} = \delta_x$, then $D_j^{2ps_j - [n/2]-1} g_{x,t}{}^{(p)}$ will be locally square-summable. In particular, if $\zeta \in C_c^\infty(N)$ with $\zeta = 1$ on a neighborhood N_1 of x, and if $ps_j \geq [n/2]+1$ for all j, then $h_{x,t} = \zeta^s g_{x,t}{}^{(p)}$ for any positive s will lie in $H_{p,t}(N)$, the completion of $C_c^\infty(N)$ with respect to the norm defined by $\|\phi\|^2_{H_{p,t}(N)} = \|\phi\|^2_{H_p(N)} + t(\phi, \phi)$, $t \geq t_p$.

If A is a formally self-adjoint semi-elliptic differential operator, its realization as an operator in $L^2(G)$ with domain $C_c^\infty(G)$ is symmetric and bounded from below. Let A_1 be one of its self-adjoint extensions which is bounded from below. (Such extensions exist.) Then, for $t \geq t_0$, there exists a distribution $g_{x,t}{}^{(p)}$ of order at most $[n/2]+1$ such that, after getting rid of the discontinuity on a null set, $((A_1^p + t)^{-1}\phi)(x) = (\phi, g_{x,t}{}^{(p)})$ for $\phi \in C_c^\infty(G)$. Similarly, if $\{E_\lambda\}$ is the spectral resolution of A_1, $(E_\lambda\phi)(x) = (\phi, e_{x\lambda})$, where $e_{x,\lambda}$ is the spectral function of A_1. The regularity properties imply that $e_{x,\lambda}$ lies in $C^\infty(G)$, $g_{x,t}{}^{(p)}$ is continuous, $g_{x,t}{}^{(p)}(y) = \int 1/(\lambda^p + t) d_\lambda e_{x,\lambda}(y)$. Further, $\|g_{x,t}{}^{(p)}\|_{L^2(N)}$ is uniformly bounded for $t \geq t_0 + 1$, $((A^p + t)\phi, g_{x,t}{}^{(p)}) = \phi(x)$ for $\phi \in C_c^\infty(G)$.

Let $s >$ order A, y the variable on N, $\phi \in C_c^\infty(N_1)$, $A_0 = \sum_{|\alpha|_m = q} a_\alpha(x) D_y^\alpha$. Then:

$$\overline{\phi(x)} = (h_{x,t}, (A_0^p + t)\phi) + (h_{x,t}, (A^p - A_0^p)\phi). \tag{1}$$

Suppose $\epsilon > 0$ is given. Then there exists $d > 0$ such that if support $(\phi) \subset N_d(x) = \{y : |y - x| < d\}$, the second term of the right-hand side of relation (1) is less than $\epsilon \|h_{x,t}\|_{H_p(N)} \|\phi\|_{H_p(N)}$. Let $H_{x,t} = F(h_{x,t})$, $v_j = (pq)^{-1} m_j$, $v = \sum_j v_j$, $\xi_j = t^{v_j}\eta_j$ (in symbols, $\xi = t^{(v)}\eta$), $a(\eta) = A_0(\eta)^p + 1$. We note that $|\phi(x)| \leq (2\pi)^{-n_2} \int |F(\phi)(\xi)|\, d\xi \leq \|\phi\|_{H_p,\, t(N)} \{\int d\xi/(A_0(\xi)^p + t)\}^{1/2}$, and, after transforming to the η-co-ordinates, the last integral equals $t^{v-1} \int d\eta/a(\eta) < \infty$.

Let H^1 be the Hilbert space of measurable functions f on R^n for which $\int a(\eta) |f(\eta)|^2\, d\eta < \infty$ with inner product $[f; g] = \int_{R^n} a(\eta) f(\eta) \overline{g(\eta)}\, d\eta$. If ψ is any element of $C_c^\infty(E^n)$, $F(\psi)$ lies in H^1. The set of such $F(\psi)$ is dense in H^1, for if $f \in H^1$ is orthogonal to all $F(\psi)$, i.e., $[f, F(\psi)] = 0$, then, translating ψ by any real vector y, $\int_{R^n} a(\eta) f(\eta)\, e^{i<y,\eta>} F(\psi)(\eta)\, d\eta = 0$. Since $a(\eta) f(\eta) F(\psi)(\eta)$ is summable, by the uniqueness of Fourier transform in L^1, $a(\eta) f(\eta) F(\psi)(\eta) = 0$ a.e. and $f(\eta) = 0$ a.e.

If $\psi \in H_{p,t}(N_d)$ and t is positive, the family of functions $\psi_t(y) = \psi(x + y/t^{(v)})$ runs through $H_{p,t}(t^{(v)}(N_d - x))$ as ψ runs through $H_{p,t}(N_d)$, and, for t sufficiently large, includes any given function in $C_c^\infty(E^n)$. Let $J_{x,t}(\eta) = te^{i<x,\, t^{(v)}\eta>} H_{x,t}(t^{(v)}\eta)$, and note that $F(\psi_t)(\eta) = t^v e^{i<x,\, t^{(v)}\eta>} F(\psi)(t^{(v)}\eta)$. Using the Parseval equality and substituting $\xi = t^{(v)}\eta$, we obtain, for $\psi \in C_c^\infty(N_1)$,

$$(h_{x,t}, (A^p + t)\psi) - \overline{\psi(x)} = [J_{x,t}, F(\psi_t)] - \left[\frac{1}{a}, F(\psi_t)\right](2\pi)^{-n/2}. \tag{2}$$

On the other hand, we have

$$\|h_{x,t}\|^2_{H_p(N)} \leq \|h_{x,t}\|_{H_p,\, t(N)} = t^{v-1}\|J_{x,t}\|^2_{H^1}, \tag{2a}$$

$$\|\psi\|^2_{H_p(N)} \leq \|\psi\|^2_{H_p,\, t(N)} = t^{1-v}\|F(\psi_t)\|^2_{H^1}. \tag{2b}$$

Thus relations (1) and (2) imply, for $\psi \in C_c^\infty(N_d)$,

$$\left|[J_{x,t}, F(\psi_t)] - \left[\frac{(2\pi)^{-n/2}}{a}, F(\psi_t)\right]\right| \leq \epsilon \|J_{x,t}\|_{H^1} \|F(\psi_t)\|_{H^1}. \tag{3}$$

Now let ϕ be any element of $C_C^\infty(N)$. We have

$$\phi(x) = ((A^p + t)(\zeta^s\phi), g_{x,t}^{(p)}) = ((A^p + t)\phi, \zeta^s g_{x,t}^{(p)}) + \sum_{\substack{|\alpha|_m + |\beta|_m \leq q \\ |\beta| > 0}} (c_\alpha D^\alpha \phi, D^\beta(\zeta^s) g_{x,t}^{(p)}).$$

Estimating the last sum, it follows that, for any $\epsilon > 0$, there exists $K\epsilon$, such that the absolute value of the sum is less than $\|\phi\|_{H_p(N)} \{\epsilon \|\zeta^s g_{x,t}^{(p)}\|_{H_p(N)} + K\epsilon \|g_{x,t}^{(p)}\|_{L^2(N)}\}$. Since $|\phi(x)| \leq ct^{v-1/2}\|\phi\|_{H_p,\, t(N)}$, while, for $t \geq t_0$, $\|h_{x,t}\|_{H_p,\, t(N)} \leq c_1 \sup_{\phi \in C_c^\infty(N)} |((A^p + t)\phi, \zeta^s g_{x,t}^{(p)})|/\|\phi\|_{H_p,\, t(N)}$, we have, for $c_1\epsilon < 1/2$,

$$\|h_{x,t}\|^2_{H_p,\, t(N)} \leq c_2 \|g_{x,t}^{(p)}\|_{L^2(N)} + c_3 t^{v-1/} \leq c_4\{1 + t^{v-1/2}\}. \tag{4}$$

In particular, $t\|g_{x,t}^{(p)}\|^2_{L^2(N_1)} \leq c_5\{1 + t^{v-1}\}$ and $\|g_{x,t}^{(p)}\|_{L^2(N_1)} \leq c_6\{t^{-1/2} + t^{v/2-1}\}$. Since the same estimate could have been carried through for N, we have, from relation (2a) above and the first inequality in relation (4), that $\|h_{x,t}\|_{H_p,\, t(N)} \leq c_7 t^{v-1/2}$ and $\|J_{x,t}\|_{H^1} \leq c_7$.

If $d > 0$ and $\psi' \in C_c^\infty(E^n)$, there exists $T > 0$ such that for $t \geq T$, $\psi' \in C_c^\infty(t^{(x)}(N_d - x))$. We then have $|[J_{x,t}, F(\psi')] - [(2\pi)^{-n/2}/a, F(\psi')]| \leq \epsilon c_7 \|F(\psi')\|_{H^1}$. Since $J_{x,t}$ has bounded norm in H^1 as $t \to \infty$, it follows that, as $t \to \infty$, $J_{x,t}$ converges weakly to $(2\pi)^{-n/2}(1/a)$ in H' But then $[J_{x,t}, 1/a] \to (2\pi)^{-n/2}\|1/a\|^2_{H^1}$, i.e., $t^{1-q}g^{(p)}_{x,t}(I) \to (2\pi)^{-n} \int d\xi/a(\xi)$. If x_0 were a point outside N, a similar argument would show that $t^{1-q}g^{(p)}_{x_0,t}(x) \to 0$. By suitable choice of N this is true for any $x_0 \neq x$. Applying a Tauberian theorem of Hardy and Littlewood,[7] we obtain

THEOREM 1. *Let $x, y \in G$. Then, as $\lambda \to +\infty$, $e_{x,\lambda}(y) = (2\pi)^{-n} w_{A_0}(x)(\delta_{x,y} + 0(1))\lambda^b$, where $b = q^{-1}(\sum_{j=1}^{n} m_j)$, $w_{A_0}(x) = \int_{A_0(x,\xi)<1} d\xi(\delta_{xy} = 1$ if $x = y$, 0 if $x \neq y$).*

A_1 is said to be regular if $(A_1 + iI)^{-1}$ is compact. In the regular case $e_{x,\lambda}(y) = \sum_{\lambda_i \leq \lambda} \overline{u_j(x)}\, u_j(y)$ (u_j an orthonormal set of eigenfunctions with eigenvalues λ_j). For the general set of elliptic variational boundary-value problems for which the writer has shown the regularity of solutions on the boundary,[8] we have $t^{1-q}|g_{x,t}{}^{(p)}(x)| \leq f(x)$, f summable. Interchanging limits by dominated convergence, we have

THEOREM 2. *Let G be a bounded domain of class C^{pq}, A_1 defined with respect to a smooth elliptic boundary-value problem on G.[8] Then $N(z) = \sum_{\lambda_j \leq t} 1 = (1 + 0(1))(2\pi)^{-n}\{\int w_{A_0}(x)\, dx\}t^b$, as $t \to +\infty$.*

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[4] F. E. Browder, *Compt. rend. Acad. sci.* (Paris), **236**, 2140–2142, 1953.

[5] L. Gårding, *Förh. Kgl. Fysiograf. Sallskap. Lund*, Vol. **21**, No. 11, 1951.

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IRREDUCIBLE OPERATOR ALGEBRAS

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1. *Introduction.*—One of the classical results of the theory of operator algebras states that each C^*-algebra (operator-bound and adjoint closed algebra of operators on a Hilbert space) has a separating family of representations as irreducible C^*-algebras (those with no *closed* invariant subspaces other than (0) and the entire space)—a corollary of which is the existence of a separating family of strongly continuous, irreducible, unitary representations of a locally compact group.

In this note we shall prove that each irreducible C^*-algebra is algebraically irreducible (has no proper invariant linear manifolds—closed or otherwise) and

develop some of its consequences.[1] We shall prove, in fact, the n-fold transitivity of irreducible C^*-algebras.

2. *Transitivity and Irreducibility.*—The main result is contained in

THEOREM 1. *If $\mathfrak{A}$ is a C^*-algebra acting irreducibly upon the Hilbert space $\mathfrak{H}$ and $\{x_i\}$, $\{y_i\}$ are two sets containing n vectors each, the first set consisting of linearly independent vectors, then there exists an operator A in $\mathfrak{A}$ such that $Ax_i = y_i$. In particular, $\mathfrak{A}$ acts algebraically irreducibly upon $\mathfrak{H}$. If $Bx_i = y_i$ for some self-adjoint operator B, then a self-adjoint operator A in $\mathfrak{A}$ may be chosen such that $Ax_i = y_i$.*

Proof: Choosing an orthonormal basis for the space generated by $\{x_i\}$, there will be a set of n vectors such that the class of operators mapping the basis onto this set coincides with the class mapping the x's onto the y's. It suffices, therefore, to deal with the case where $\{x_i\}$ is an orthonormal set.

We note that if $z_1, \ldots, z_n$ are vectors such that $\|z_i\| \leq r$, there is an operator B of norm not exceeding $(2n)^{1/2}r$ (and self-adjoint, in case $z_i = Tx_i$, for some self-adjoint operator T) such that $Bx_i = z_i$. In fact, let $x_1, \ldots, x_n, x_{n+1}, \ldots, x_m$ be an extension of the set $x_1, \ldots, x_n$ to an orthonormal basis of the space $\mathfrak{V}$ generated by $x_1, \ldots, x_n, z_1, \ldots, z_n$. With T and S linear transformations on $\mathfrak{V}$, the mapping which assigns trace (S^*T) to the pair (T, S) defines an inner product and hence a norm—the norm, $[T] = [\text{trace } (T^*T)]^{1/2}$, of T is $(\sum_{i,j} |\alpha_{ij}|^2)^{1/2}$, where the α_{ij}'s are the matrix coordinates of T relative to any orthonormal basis of $\mathfrak{V}$. The matrix whose first n columns (and conjugate first n rows, in the self-adjoint case) are $z_1, \ldots, z_n$—or, rather, their co-ordinate representation, relative to $x_1, \ldots, x_m$—and zeros elsewhere represents an operator B such that $Bx_i = z_i$, and $[B] \leq (2n)^{1/2}r$. It is an elementary fact, however, that $\|B\| \leq [B]$, where $\|B\|$ denotes the usual operator bound of B. Defining B to be 0 on the orthogonal complement of $\mathfrak{V}$, we have an operator with the desired properties.

We proceed now to the construction of an operator A in $\mathfrak{A}$ such that $Ax_i = y_i$. Choose B_0 such that $B_0x_i = y_i$, and A_0 in $\mathfrak{A}$ such that $\|A_0x_i - B_0x_i\| = \|A_0x_i - y_i\| \leq [2(2n)^{1/2}]^{-1}$. (Recall that since $\mathfrak{A}$ is irreducible, its strong closure is the set of all bounded operators on $\mathfrak{H}$.) Next choose B_1 such that $B_1x_i = y_i - A_0x_i$, with $\|B_1\| \leq 1/2$. Kaplansky's theorem[2] guarantees the existence of an operator A_1 in $\mathfrak{A}$ with $\|A_1\| \leq 1/2$ such that $\|A_1x_i - B_1x_i\| \leq [4(2n)^{1/2}]^{-1}$. Suppose now that B_k has been constructed with $\|B_k\| \leq 1/2^k$, $B_kx_i = y_i - A_0x_i - A_1x_i - \ldots - A_{k-1}x_i$. Choose A_k in $\mathfrak{A}$ with $\|A_k\| \leq 1/2^k$ such that $\|A_kx_i - B_kx_i\| \leq \dfrac{1}{2^{k+1}(2n)^{1/2}}$, and B_{k+1} with $\|B_{k+1}\| \leq 1/2^{k+1}$, $B_{k+1}x_i = y_i - A_0x_i - \ldots - A_kx_i$. Note that if $Tx_i = y_i$, for some self-adjoint operator T, the results of the preceding paragraph allow us to choose self-adjoint B_k's and hence, by Kaplansky's theorem, self-adjoint A_k's. The sum $\sum_{k=0}^{\infty} A_k$ converges in norm to an operator A in $\mathfrak{A}$, and

$$y_i - Ax_i = y_i - \sum_{k=0}^{\infty} A_kx_i = \lim_k (y_i - A_0x_i - \ldots - A_kx_i) = \lim_k B_{k+1}x_i = 0.$$

3. *States and Ideals.*—A state of a C^*-algebra is a linear functional which is 1 at the identity operator I and non-negative on positive operators. The set of states of a C^*-algebra is a convex set whose extreme points generate the set. These extreme points are called "pure states." Each state ρ of $\mathfrak{A}$ gives rise to a representation[3] φ of $\mathfrak{A}$ by means of the following process. The set of elements A in $\mathfrak{A}$ such

that $\rho(A^*A) = 0$ is a left ideal $\mathfrak{s}$ in $\mathfrak{A}$—the so-called "left kernel of ρ"—it is the largest left ideal in the null space of ρ. Let $\mathfrak{H}_0$ be the vector-space quotient $\mathfrak{A}/\mathfrak{s}$. The mapping which assigns $\rho(B^*A)$ to the pair $(A + \mathfrak{s}, B + \mathfrak{s})$ is well defined and a positive-definite bilinear form on $\mathfrak{H}_0$, so that the completion $\mathfrak{H}$ of $\mathfrak{H}_0$ relative to the metric induced by this inner product is a Hilbert space. The operator $\varphi(A)$ defined on $\mathfrak{H}_0$ by $\varphi(A)[B + \mathfrak{s}] = AB + \mathfrak{s}$ is continuous and has a unique extension as a bounded operator on $\mathfrak{H}$ (which we again denote by $\varphi(A)$). The mapping $A \to \varphi(A)$ is the desired representation of $\mathfrak{A}$ as a C^*-algebra. It is well known[3] that ρ is a pure state if and only if $\varphi(\mathfrak{A})$ is an irreducible C^*-algebra. With the notation just established, we can state

COROLLARY 1. *If ρ is a pure state, $\mathfrak{A}/\mathfrak{s}$ is complete relative to the inner product induced by ρ.*

Proof: From Theorem 1, $\varphi(\mathfrak{A})$ acts algebraically irreducibly upon $\mathfrak{H}$, whence $\mathfrak{H}_0 = \{\varphi(A)[I + \mathfrak{s}]: A \text{ in } \mathfrak{A}\} = \mathfrak{H}$.

COROLLARY 2. *The null space $\mathfrak{N}$ of ρ is $\mathfrak{s} + \mathfrak{s}^*$ if and only if ρ is a pure state.*

Proof: If $\mathfrak{N} = \mathfrak{s} + \mathfrak{s}^*$ and $\rho = (\rho_1 + \rho_2)/2$, with ρ_1 and ρ_2 states of $\mathfrak{A}$, then, for A in $\mathfrak{s}$, $0 = \rho(A^*A) = \rho_1(A^*A) = \rho_2(A^*A)$; and $\rho_1(A) = \rho_2(A) = 0$, by Schwarz's inequality. Thus ρ_1 and ρ_2 annihilate $\mathfrak{N}$, and $\rho_1 = \rho_2 = \rho$, so that ρ is a pure state.

Assume now that ρ is a pure state. In the Hilbert space $\mathfrak{A}/\mathfrak{s}$, the subspace $\mathfrak{N} + \mathfrak{s}$ is the orthogonal complement of the vector $I + \mathfrak{s}$. The ideal $\mathfrak{s}$ is the set of elements A in $\mathfrak{A}$ such that $\varphi(A)$ annihilates this same vector. With B in $\mathfrak{N}$, Theorem 1 tells us that there exists a self-adjoint operator $\varphi(A)$ in $\varphi(\mathfrak{A})$ (so that A may be chosen self-adjoint) which annihilates $I + \mathfrak{s}$ and leaves $B + \mathfrak{s}$ fixed; i.e., A lies in $\mathfrak{s}$ and $AB - B = -C$, with C in $\mathfrak{s}$. If B is self-adjoint, then $B = C^* + BA$, so that B lies in $\mathfrak{s} + \mathfrak{s}^*$. Since $\mathfrak{N} = \mathfrak{N}^*$, it follows that $\mathfrak{s} + \mathfrak{s}^*$ contains $\mathfrak{N}$. Clearly, $\mathfrak{N}$ contains $\mathfrak{s} + \mathfrak{s}^*$, whence $\mathfrak{N} = \mathfrak{s} + \mathfrak{s}^*$.

THEOREM 2. *The left kernel $\mathfrak{s}$ of ρ is a maximal left ideal in $\mathfrak{A}$ if and only if ρ is a pure state, in which case ρ is the unique state whose null space contains $\mathfrak{s}$. Each closed left ideal in $\mathfrak{A}$ is the intersection of the maximal left ideals containing it.*

Proof: If ρ is a pure state, $\varphi(\mathfrak{A})$ acts algebraically irreducibly upon $\mathfrak{A}/\mathfrak{s}$, and a proper left ideal containing $\mathfrak{s}$ would give rise to a proper, invariant, linear manifold in $\mathfrak{A}/\mathfrak{s}$ under $\varphi(\mathfrak{A})$. Thus $\mathfrak{s}$ is maximal.

If $\mathfrak{L}$ is a closed left ideal and $\mathfrak{K}$ a left ideal (possibly $\mathfrak{A}$) containing $\mathfrak{L}$ which is annihilated by each state which annihilates $\mathfrak{L}$, then $\mathfrak{K} = \mathfrak{L}$. In fact, with A a positive operator in $\mathfrak{K}$, the set S of states at which A is not less than $1/n^2$ is w^*-compact. The hypothesis, compactness, and the definition of the w^*-topology guarantee the existence of a finite open covering $\{U_i\}$, $i = 1, \ldots, m$, of S and elements A_i in $\mathfrak{L}$ such that A_i (and hence $A_i^*A_i$, by the Schwarz inequality) does not vanish on U_i. Some positive multiple T_n^2 of $A_1^*A_1 + \ldots + A_m^*A_m$ exceeds A on S, whence $T_n^2 + I/n^2 \geq A$, where T_n, the positive square root of T_n^2, being a uniform limit of polynomials in T_n^2 without constant terms, by the Weierstrass approximation theorem, lies in $\mathfrak{L}$. Now

$$\|A^{1/2}(T_n + I/n)^{-1}T_n - A^{1/2}\|^2 = \|(T_n + I/n)^{-1}A(T_n + I/n)^{-1}\|/n^2 \leq \|(T_n + I/n)^{-2}(T_n^2 + I/n^2)\|/n^2 \leq 1/n^2.$$

Thus $A^{1/2}$, and hence A, lies in $\mathfrak{L}$. With B an arbitrary element in $\mathfrak{K}$, B^*B and $(B^*B)^{1/2}$ lie in $\mathfrak{L}$, from the above, while[4]

$$\|B[(B^*B)^{1/2} + I/n]^{-1}(B^*B)^{1/2} - B\| \leq 1/n,$$

as above, so that B lies in $\mathfrak{L}$ and $\mathfrak{K} = \mathfrak{L}$.

From the foregoing, it follows, in particular, that if $\mathfrak{L}$ is a proper ideal, the set $\mathfrak{S}$ of states which annihilate $\mathfrak{L}$ is non-null—this set is convex and w^*-compact and so the closed, convex hull of its set $\mathfrak{E}$ of extreme points. The points of $\mathfrak{E}$ are trivially seen to be extreme in the set of all states, i.e., pure states, and the hull property guarantees that an operator which annihilates $\mathfrak{E}$ annihilates $\mathfrak{S}$. The intersection of the left kernels of states in $\mathfrak{E}$ is a left ideal, containing $\mathfrak{L}$ and annihilating $\mathfrak{S}$, so that this intersection is $\mathfrak{L}$. The first paragraph of the proof assures us, however, that these left kernels are maximal left ideals, and the last assertion of the theorem is proved.

If $\mathfrak{g}$ is a maximal left ideal, there is a pure state η which annihilates it and for which it must therefore be the left kernel. Corollary 2 tells us that $\mathfrak{g} + \mathfrak{g}^*$ is the null space of η, hence of ρ, so that $\rho = \eta$ and the proof is complete.

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[1] Few technicians would have entertained seriously the possibility of the truth of the results contained in Theorem 1 and Corollary 1, and even the validity of the results of Corollary 2 and Theorem 2 seemed highly doubtful. R. Prosser has been developing a duality theory for C^*-algebras, by topological linear space methods, which, when complete, should put the results of Theorem 2 in its appropriately general setting. It was the convincing nature of Prosser's program which led the author to re-examination of this area and thence to the results of this note. The author wishes to record his gratitude to R. Prosser for many stimulating discussions about operator algebras.

[2] I. Kaplansky, "A Theorem on Rings of Operators," *Pacific J. Math.*, **1**, 227–232, 1951.

[3] I. Segal, "Irreducible Representations of Operator Algebras," *Bull. Am. Math. Soc.*, **53**, 73–88, 1947.

[4] In essence, this inequality establishes that a closed left ideal is generated by its positive elements—a fact which seems to have been proved first by I. Segal in "Two-sided Ideals in Operator Algebras," *Ann. Math.*, **50**, 856–865, 1949, using a similar estimate.

ZEROS OF NEIGHBORING HOLOMORPHIC FUNCTIONS

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1. *A Lemma.*—The functions $g(z)$ and $f(z)$ are holomorphic throughout the closed disk $\Delta_\rho = \{z: |z - \zeta_0| \leq \rho\}$ on which $f(z)$ has the sole zero ζ_0, and that of order $p \geq 1$. Let

$$0 < m \leq |f(\zeta_0 + \rho e^{i\theta})| \leq M \qquad (0 \leq \theta \leq 2\pi), \tag{1}$$

and define

$$\|f - g\|_\rho = \max_{z \in \Delta_\rho} |f(z) - g(z)|. \tag{2}$$

So soon as it satisfies the inequality $\|f - g\| < m$, $g(z)$ *is assured of having exactly* p

zeros (counting multiplicities) $\zeta_1, \zeta_2, \ldots, \zeta_p$ on Δ_ρ. Some, say l, of these zeros may coincide with ζ_0: $\zeta_{p-l+1} = \zeta_{p-l+2} = \ldots \zeta_p = \zeta_0$. *The* $p - l$ *zeros* $\zeta_1, \zeta_2, \ldots, \zeta_{p-l}$ *which differ from* ζ_0 *verify the inequalities*

$$\frac{\rho}{M+m}\left|\frac{g^{(l)}(\zeta_0)}{l!}\right| \leq |\zeta_i - \zeta_0| \leq \left(\frac{\|g-f\|^{1/p}}{m}\right)\rho, \tag{3}$$

$$\frac{\rho^{p-l}}{M+m}\left|\frac{g^{(l)}(\zeta_0)}{l!}\right| \leq \prod_{i=1}^{p-l}|\zeta_i - \zeta_0|, \tag{4}$$

where $i = 1, 2, \ldots, p - l$.

The inequality on the right in relation (3) is known[1] and follows immediately from equation (2) and $f(\zeta_i) = (\zeta_i - \zeta_0)^p \varphi(\zeta_i) = f(\zeta_i) - g(\zeta_i)(i = 1, 2, \ldots, p)$. Relation (4) is Jensen's inequality—or can also be derived directly—and leads to the left-hand inequality in relation (3). This lemma removes the restriction to simple zeros considered in a previous note.[2]

2. *Speed of Convergence of the Zeros of a Sequence of Functions.*—A straightforward consequence of inequalities (3) and (4) is

THEOREM 1. ζ_0 *being a p-fold zero of the function* $f(z)$ *holomorphic in the disk* Δ_ρ *where* ζ_0 *is its only zero, let the functions* $g_n(z)$ $(n = 1, 2, \ldots)$, *holomorphic in* Δ_ρ *and verifying* $g_n(\zeta_0) \neq 0$, *converge uniformly to* $f(z)$ *in* Δ_ρ. *If* $\{\alpha_n\}$ *is a monotone decreasing sequence of positive numbers converging to zero, for which the relations*

$$\overline{\lim_n}\ |g_n(\zeta_0)|^{\alpha_n} = A \qquad \text{and} \qquad \overline{\lim_n}\,(\|g_n - f\|_\rho^{\alpha_n}) \leq A(\rho) \tag{5}$$

hold (where $A(\rho) \geq A$ *and* $A(\rho) \to A$ *as* $\rho \to 0$*), then the following relations are true:*

$$\overline{\lim_n}\,(|\zeta_{nj} - \zeta_0|^{p\alpha_n}) = A \qquad (j = 1, 2, \ldots, p). \tag{6}$$

Here $\zeta_{n1}, \zeta_{n2}, \ldots, \zeta_{np}$ *are the p zeros of* $g_n(z)$ *whose presence in* Δ_ρ *is guaranteed for all sufficiently large n by a classical theorem of Hurwitz.*

Note that the condition $g_n(\zeta_0) \neq 0$ can be relaxed to: ζ_0 is a zero of exact order $l < p$ of $g(z)$. The theorem then applies to the function $f(z)/(z - \zeta_0)^l$ and the sequence $\{g_n(z)/(z - \zeta_0)^l\}_n$.

3. *Zeros of Partial Sums of Dirichlet Series and Taylor Series.*—Direct, though too lengthy to reproduce here, application of Theorem 1 yields the following:

Let $f(s) = \sum_{i=0}^{\infty} a_i e^{-\lambda_i s}$ $(0 \leq \lambda_0 < \lambda_1 < \ldots < \lambda_i < \ldots)$ *have the abscissa of convergence* $c < \infty$, *and let* $\zeta_0 = R\zeta_0 + iI\zeta_0$ *be a p-fold zero of* $f(s)$ *in the interior of its half-plane of convergence. Then, setting* $g_n(s) = \sum_{i=0}^{n} a_i e^{-\lambda_i s}$, *and designating by* $\zeta_{nj}(j = 1, 2, \ldots, p)$ *the p zeros of* $g_n(s)$ *which tend to* ζ_0 *as* $n \to \infty$, *we have*

$$\overline{\lim_n}\left(\frac{\log|\zeta_{nj} - \zeta_0|}{\lambda_n}\right) = \frac{c - R\zeta_0}{p} \quad (j = 1, 2, \ldots, p). \tag{7}$$

The obvious analogue for Laplace transforms can be shown in the same way.

The properties derived by S. Doss[2] for the zeros of partial sums of Taylor series, linking their speed of convergence to the radius of convergence of the series or to the order of the function, can also be shown to follow from Theorem 1.

[1] S. Doss, "Comportement asymptotique des zéros de certaines fonctions d'approximation," *Ann. École norm. supér.*, **3** (64), 143, 1947.

[2] T. P. G. Liverman, "Implicit Function Theorem for Quasi-analytic and Related Classes of Functions," these PROCEEDINGS, **42**, 261–264, 1956.

A PRELIMINARY NOTE ON EXCITATION-CONTRACTION COUPLING

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When a short-lived action potential passes along the muscle fiber membrane, contraction invariably follows. The mechanism whereby the contractile system is activated up to a distance as great as 50 μ from the membrane, and within 30 msec., is unknown (Hill,[1] Katz,[2] and Sandow[3]).

Although currents of considerable strength flow along the interior of the fiber during membrane activity, some workers have concluded that these currents are ineffective in setting off the active state (for references see Sten-Knudsen[4]). It is claimed to be clear that the essential thing is the change in the potential difference across the membrane itself (Huxley[5]). These conclusions are based on the following findings (see Sten-Knudsen[4]). When muscle is quickly immersed in excess-K Ringer, the fiber membrane is depolarized, but no internal currents flow. Under these conditions, nevertheless, tension develops in the form of contracture. When muscle is rendered nonpropagating by procaine or by Na lack, only its cathodal end responds to a longitudinal d.c. field. Muscle rendered nonpropagating by excess K develops practically maximum tension in a transverse field and almost no tension in a longitudinal field. When two microelectrodes are inserted in a single muscle fiber, rendered nonpropagating by procaine or by Na lack, and a longitudinal current is generated between the two electrodes, contraction occurs only if the two electrodes are so far apart that current escapes from the fiber, leaving the membrane depolarized (Watanabe and Ayabe, personal communication).

In addition to these findings, evidence is now available that the influence of membrane depolarization is conveyed to the interior of the fiber by spread along the "Z" bands (Huxley and Taylor[6]).

These observations point to the importance of depolarization in the series of events leading to activation, without demonstrating that depolarization alone can accomplish the task. The effect of depolarization is known to be localized in the near vicinity of the membrane, and in case of radial propagation its effect cannot exceed the close neighborhood of the "Z" bands. The distance to be covered by the coupling mechanism between two "Z" bands is 2.5 μ or more. In smooth muscle there is no cross-striation at all, and thus it remains to be explained how the effect of depolarization is "felt" by the contractile system in muscles where no "Z" bands are available for radial propagation.

Our experiments, to be reported elsewhere in detail, suggest that depolarization

is only a "priming" first step in the series of events leading to activation and that internal currents effectively contribute to the activation process. This conclusion is based on a series of experiments performed on frog twitch muscles (sartorius and m. ext. long. dig. IV). When muscle is made to develop contracture in excess K, tension is never maximal, but fractional. Only partial tension develops, even if quick "inactivation," i.e., the release of contracture, is effectively delayed. However, when the muscle is in a temporary "steady-state" partial contracture, it develops maximum tension (superimposed upon the contracture) if stimulated in an effective longitudinal a.c. field. Clearly depolarization alone is relatively ineffective, whereas the combined effect of depolarization and the field produces almost maximum tension.

Muscle rendered nonpropagating by excess K can be effectively activated in a longitudinal a.c. field if the field strength is adequate, the frequency is optimal, and the membrane is kept depolarized within the critical range of 50–42 mv. Within this critical range of membrane potential, which can be widened by excess Ca, membrane potential and tension are inversely related. Correcting for the effect of Ca on the membrane potential (Jenerick and Gerard[7]), it can be shown that, in the nonpropagating muscle, tension at a constant membrane potential is a function of the $[Ca]_0$. This, together with various facts ascertained by others (for references see Sandow[3]), suggests that Ca is a key ion in excitation-contraction coupling.

The fact that nonpropagating muscle is effectively activated in an external longitudinal a.c. field cannot, however, be used as evidence for the effectiveness of internal currents in activation. It may be argued that the effect of the external field is due to electrotonic membrane-potential changes, which can spread from the ends toward the middle of the fiber if the field is sufficiently strong. Or it may be said that the effect is associated with depolarization (in addition to the effect of excess K), resulting from changes in the longitudinal resistance of the fiber, due to irregularities.

The electrotonic membrane-potential change is clearly greatest at the ends, and at the threshold only the ends should be involved in the change. From this it follows that at the threshold only the ends of a nonpropagating muscle should contract if the effect is due to electrotonic membrane-potential change. One can therefore expect a concave curve when the extent of shortening is plotted against the order of segments along the muscle length, demonstrating that the ends contract more than the middle. On the other hand, if activation is associated with depolarization resulting from diversion of the longitudinal current, due to irregularities in fiber diameter or in the space between the fibers, shortening along the length of the muscle should be irregular.

Our experiments showed that neither of these predictions is valid. When shortening of differing segments was plotted against the order of segments along the length of the nonpropagating muscle, a family of *convex* rather than concave or irregular curves was obtained. The curves shifted upward on the shortening axis as the field strength in the longitudinal field was increased. It was the middle which contracted most and first, and at the threshold only the middle contracted. The curves remained convex when cross-sectional asymmetry was corrected for by

plotting shortening (shortening in the nonpropagating/shortening in the propagating muscle × 100) against the order of segments.

This finding, in the light of the observations of Rushton,[8] Hodgkin and Rushton,[9] and Katz,[10] can be taken as evidence that the effect of the external longitudinal field is associated with internal currents. These authors demonstrated that "the voltage gradient along the inside of the fibers is the smallest at the ends and the greatest along the middle portion" (Sten-Knudsen[4]).

In our experiments shortening at different portions along the muscle length was measured photographically. The sartorius and toe muscles of the frog (*Rana pipiens*) were marked off into six segments of approximately equal length by transverse nylon threads or graphite particles. The normal and the nonpropagating muscles were made to develop isotonic work in normal and excess-K (16 mM) Ringer by the application of a longitudinal a.c. field of varying strength and of low frequency (60 c/s). Threshold and optimum stimuli were determined by the magnitude of the mechanical response. The intactness of the muscle at the end of the experiment was assured by the return of the normal propagated response and maximum tension, after repolarization in normal Ringer. The extent of shortening along the muscle was determined by comparing resting length with shortened length at the peak of isotonic work or by taking a series of motion pictures at 20-msec. intervals. The use of a d.c. stimulus must be avoided in these experiments because it hyperpolarizes one end of the muscle and also leads to irreversible changes.

These experiments clarified the following points. The nonpropagating muscle shortens along its whole length, and not only at its ends, in a longitudinal a.c. field. When the stimulus was optimal, shortening and tension were close to maximal. At the threshold stimulus, only the middle portion of the muscle contracted and not the ends. When shortening of different segments was plotted against the order of segments along the muscle length, a *convex* (rather than concave or irregular) curve was obtained. Increasing the field strength resulted in an increase in shortening of all segments, and the convex curve plotted from the threshold response shifted upward on the shortening axis, without much change in shape. This and the fact that the toe muscle required a weaker stimulus than the sartorius for the same effect illustrate that the requirement of a strong current for maximum effect in the nonpropagating sartorius muscles (20 v/cm) is explained by poor current penetration toward the depth of the muscle. Some experiments also suggest that the middle of the muscle begins to shorten first.

It may be argued that greater shortening in the middle, rather than at the ends, of the muscle is due to an asymmetry in the cross-sectional area at different portions of the muscle. When relative shortening (shortening nonpropagating/shortening propagating muscle × 100) is plotted against the order of segments, however, cross-sectional asymmetry can be corrected for. The shape of such a curve remains convex, illustrating that preferential shortening in the middle portion is a genuine effect. This suggests that the effect of the external longitudinal field on the shortening of the nonpropagating muscle is associated with effective internal currents. This is significant because during normal excitation the longitudinal component of the action potential creates a considerable electric field ($\cong$3 v/cm) in the interior of the fibers. It is also established that the time integral of the internal field is uniform across the whole cross-sectional area of the fiber (Bay, Goodall, and Szent-Gyorgyi[11]).

These observations, together with earlier findings, suggest the following tentative mechanism for excitation-contraction coupling. The process is started off by the propagated action potential, which eliminates the potential difference across the fiber membrane and also penetrates radially along the "Z" bands, resulting in changes of charge density in the close vicinity of these structures. This "priming" step is an indispensable agent (Kuffler[12] and Hill[1]) in the series of events leading to contraction, but it is not adequate by itself to set up maximum activity, since its effective range is narrow. Assuming a periodic distribution of Ca and assuming that ionized Ca is instrumental in the transformation of a resting to an active muscle (for references see Sandow,[3] Weber and Portzehl,[13] and Morales and Botts[14]), it is tempting to visualize the effect of depolarization as the release of ionized Ca in the close vicinity of the fiber membrane and the "Z" bands.

The role of the internal currents in this picture is to move ionized Ca with great rapidity over a distance of 2.5 μ (i.e., between two "Z" bands) and within a period of 30 msec. (i.e., the time limit of Hill[15] for maximum activity). This movement of the Ca ion, which is known to be a powerful activator of actomyosin (as an ATP-ase), can serve to establish direct contact between this ion and the enzyme. The same may be true for Mg and the "relaxing factors" (Weber and Portzehl[13]). Calculations show that Ca can in fact move a distance of about 2 μ within a period of 30 msec., in a field of 3 v/cm. Although the validity of this picture remains to be proved, it nevertheless appears plausible.

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*THE PROBLEM OF DOMINANT LETHALS IN DROSOPHILA MELANOGASTER FEMALES**

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The dose-response relations for dominant lethal mutations induced in *Drosophila melanogaster* oöcytes of different stages have been studied by Parker[1] and by King, Darrow, and Kaye.[2] The dose-response relation for the mature primary ovarian oöcyte (stage 14) can be described by a one-event curve; that for the immature stage 7 oöcyte, by a multiple-event curve.

The one-event reactions so far analyzed[3] are generally based on the assumption that the critical event is a single ionization or the production of a small ion cluster. It does not seem very likely, however, that a single ionization or a small cluster can produce a dominant lethal, if dominant lethal mutations result from multiple isochromatid breakage. A more likely candidate is the tail end of an electron track, because in the case of an electron tail there is a considerable amount of energy (about 20 kev) released within a small tissue volume.

From the 37 per cent survival dose and a knowledge of the distribution of initial energy among Compton recoil electrons produced by the radiation, we can make a calculation of the diameter of the target (assumed to be spherical) in the stage 14 oöcyte. The 37 per cent survival dose for stage 14 oöcytes treated with Co^{60} gamma rays is 1.05 kr.[2] Cormack and Johns[4] have calculated that 100 r of Co^{60} gamma rays set in motion 9.94×10^9 electrons per cubic centimeter of water. The number of original electrons produced at too low an energy to qualify as tails must be essentially negligible. If one assigns the target a density equal to that of protein (1.35) and assumes the equivalence of water and protein on a weight basis, it can be calculated that 1.05 kr will produce 1.4×10^{11} electron tails per cubic centimeter of protein. The target volume is the reciprocal of this number, or 7.1 μ^3. On the other hand, if one assumes that the "hit" equals the occurrence of any cluster of ionizations in the target and that one ionization of the cluster is sufficient to produce an effect, then the calculation shows that 1.05 kr produce 8.25×10^{14} clusters (averaging 3 ionizations) per cubic centimeter of protein. The formal target volume is now 0.0012 μ^3. The volume of the karyosphere in the stage 14 oöcyte is 2.69 $\mu^3 \pm 0.27$ ($s_{\bar{x}}$, $n = 20$). More energetic electrons will make some delta rays, which will also result in electron tails, and therefore the value 1.4×10^{11} electron tails probably represents an underestimate. The discrepancy between 7.1 and 2.7 μ^3 would suggest that the total number of electron tails per cubic centimeter is about 2.6 times the original number of electrons set in motion. At any rate, it is obvious that the model afforded by the "tail" hypothesis gives a formal target diameter in much closer agreement with the cytological target diameter. We conclude that any electron tail traversing the karyosphere results in a dominant lethal.

The question now arises as to the mechanism of dominant lethal production in *Drosophila* females. It is presumed from the genetic studies of Sturtevant and

Beadle[5] on *Drosophila* and the cytological studies of Whiting[6] on *Habrobracon* and Carson[7] on *Sciara* that anaphase I dicentric chromatid bridges are eliminated by being automatically passed into polar-body nuclei. Second-division bridges cause the female pronucleus to receive an aneuploid complement of chromosomes, and this is generally lethal. We assume that whenever two of the four haploid nuclei which result from the meiotic divisions are joined by a chromatid bridge, one of the free nuclei functions as the female pronucleus. The reason for this assumption will be discussed further on. If both pairs of haploid nuclei are joined by bridges, the "probability of escape" is zero, and the result is a dominant lethal.

Thus the minimum requirements for a dominant lethal are (1) the occurrence of an isochromatid break in each of two different bivalents, (2) sister fusion of the broken chromatids, and (3) the passage of the fused bivalents so produced to opposite poles at anaphase I. A "hit" is defined as a break followed by fusion of sister chromatids. We wish to derive the probability, S_n, of the genetic material surviving exactly n hits. Since at least two hits are needed for a lethal effect, when $n = 1$, $S_n = 1$. A second hit can occur in the bivalent already hit (in this case survival is certain), or it can occur with equal probability in the other bivalent of the tetrad (this gives a chance of survival of zero). Finally, the second hit can occur in a bivalent nonhomologous to the one previously hit. In this case the second broken bivalent can go at anaphase I to the same pole (which means survival) or to the opposite pole (which means a dominant lethal). Both contingencies are equally likely, if one assumes that selection does not operate at the chromosomal level. It therefore turns out that the presence or the absence of a dominant lethal after the second hit are equally likely; hence $S_n = 0.5$. We assume that chiasmata are terminalized.

The argument is easily generalized. Suppose that the genetic material has received n hits and is going to survive with probability S_n. If an $(n + 1)$th hit is scored, it may hit a bivalent which has already been hit, in which case the probability of survival remains unchanged; or it can hit the other bivalent of the tetrad already hit, in which case the survival chance will drop to zero. Or the $(n + 1)$th hit may affect a bivalent not homologous to any that has been previously hit, in which case (by the same reasoning as before) half the time S_n remains unchanged and half the time it will drop to zero. Summing over all cases, we see that in 50 per cent of all instances $S_{n+1} = S_n$, and in 50 per cent $S_{n+1} = 0$, which means that $S_{n+1} = 0.5S_n$ for any $n > 1$. Since $S_n = 1.0$ if $n = 1$, we get $S_n = (\frac{1}{2})^{n-1}$

If x is the average number of hits in the genetic material, then the chance, P_n, of exactly n hits is given by the equation $P_n = x^n e^{-x}/n!$ Let the probability of survival, S_n, be a function of n. The probability, S_x, of surviving a dose which produces an average of x hits is given by the equation $S_x = \sum_{n=0}^{n=\infty} P_n S_n$. The probability of survival, S_n, after n hits is 1 if $n = 0$ and $(\frac{1}{2})^{n-1}$ if $n \geqq 1$. The total probability is therefore

$$S_x = e^{-x} + \left\{ \sum_{n=1}^{n=\infty} \frac{x^n e^{-n}}{n!} \cdot (\tfrac{1}{2})^{n-1} \right\},$$

$$S_x = e^{-x} + 2e^{-x} \sum_{1}^{\infty} (\tfrac{1}{2})^n \frac{x^n}{n!} = e^{-x} + 2e^{-x} \sum_{1}^{\infty} \frac{(x/2)^n}{n!}.$$

Note that

$$e^x = 1 + x + \frac{x^2}{2!} + \frac{x^3}{3!} + \ldots + \frac{x^n}{n!} \quad \text{or} \sum_0^\infty \frac{x^n}{n!}.$$

Therefore,

$$S_x = e^{-x} + 2e^{-x} \cdot [e^{x/2} - 1]$$
$$= 2e^{-x/2} - e^{-x} = 1 - (1 - e^{-x/2})^2.$$

The above equation is equivalent to a classical two-target dose-response relation having the equation $S_D/S_0 = 1 - (1 - e^{-aD})^2$. The double-target model specifies that a response will occur when each of two targets has been hit at least once, where a is the sensitivity of *each* target and D is the dose. It is obvious that $a = x/2$. For the data given in the paper by King, Darrow, and Kaye,[2] $a = 0.25$ kr^{-1}. Since there are two targets, the total sensitivity of the genetic material is 0.5 kr^{-1}, a value roughly one-half that obtained for the sensitivity of the genetic material of stage 14 oöcytes (0.95 kr^{-1}). Under the assumption that the total number of electron tails per cubic centimeter of tissue is about 2.6 times the original number of electrons set in motion by Co^{60} gamma rays, the volume of a single stage 7 target is 0.65 μ^3. The stage 7 nucleus has a volume of 910 μ^3, and the karyosome within the nucleus has a volume of 12 μ^3. It follows that the targets are units within the nucleus with dimensions smaller than the karyosome.

The above line of reasoning assumes that whenever two of the four haploid nuclei which result from the meiotic divisions are joined by a chromatid bridge, one of the free nuclei functions as the female pronucleus. On the other hand, if we assume that the female pronucleus is predetermined, the chance of escape, P_n, is equal to $(\frac{1}{2})^n$. In this case $S_x = \sum_{n=0}^{n=\infty} (\frac{1}{2})^n (x^n e^{-x}/n!) = e^{-x/2}$. According to this calculation, a single-event dose-response relation having the equation $S_D/S_0 = e^{-aD}$ should be generated. Since the stage 7 data fit a two-target curve, the determined female pronucleus model must be abandoned.

In the case of the stage 14 oöcyte a single-event dose-response curve is obtained. In the stage 14 oöcyte the chromatin is consolidated into a compact karyosphere, and it is assumed that the passage of a single electron tail through the karyosphere always causes many isochromatid breaks which undergo sister fusion. Most of these breaks involve different bivalents, and there are so many that some always pass to opposite poles at anaphase I. Therefore, any hit kills, and the stage 14 target volume should correspond to the volume of the karyosphere. The twofold difference between the total target volumes of stage 14 and stage 7 oöcytes is perhaps due to the way the target material is organized in the two cases. It may be that broken chromatids rejoin in the original configuration half the time and undergo sister union the other half in the case of the stage 7 oöcyte. Coiled bivalents may be under tension in the karyosphere. In this event an isochromatid break would result in the broken bivalents springing apart. Sister union would thus be favored.

Summary.—A model is presented which explains the present data on dominant lethal mutations in *D. melanogaster* females. Under the assumption that any one of the four haploid nuclei (which result from the meiotic divisions) can function as

the female pronucleus, the minimum requirements for a dominant lethal are (1) the occurrence of an isochromatid break in each of two different bivalents, (2) sister fusion of the broken chromatids, and (3) the passage of the fused bivalents so produced to opposite poles at anaphase I. Under these conditions the female pronucleus will always receive an aneuploid chromosome complement. Multiple isochromatid breakage may require multiple events if the chromatin is diffuse or a single event if the chromatin is compact. Evidence is presented to support the view that the female pronucleus is not predetermined.

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THREE DIPLOID CHROMOSOME NUMBERS OF MAN

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ATOMIC BOMB CASUALTY COMMISSION

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A number of authors[1, 2] have come to the conclusion that the diploid chromosome number of man is 48. While this number seems to have been well supported by their observations of 48 chromosomes in the spermatogonia, it was questioned recently by Tjio and Levan,[3] who observed metaphases clearly showing 46 chromosomes in embryonic tissues. They hesitated, however, to formulate the general statement that the correct diploid number of man is 46, since they had not studied the chromosome number in the spermatogonia. Thus it seems necessary to reinvestigate the chromosome number in the human gonad to see whether the previously accepted diploid number has to be revised as Tjio and Levan suspected.[8]

The present author has for some time studied the chromosomes in the testes of Japanese and has found surprisingly that three different diploid numbers exist in man instead of one, as had hitherto been believed. The present report describes the findings in a brief and tentative manner, leaving the details and a full discussion of various cytogenetic problems to later communications.

Materials and Methods.—The main sources of the testes used for the present studies were individuals operated on in Japanese hospitals on account of epididymitis. From each patient two pieces of the testis were taken at the time of epididymectomy and instantly fixed according to the following two methods: (I) One piece was treated first with a 0.4 per cent sodium chloride solution for about 30–60 minutes and then with a fresh mixture of 1.5 per cent chromic acid solution, formalin, and glacial acetic acid in the ratio 10:4:1 (same as Navaschin's formula) for about 4 hours. (II) The other piece was pretreated with a mixture of equal volumes of 0.5 per cent chromic acid and 1.5 per cent potassium bichromate solutions for about $1^1/_2$ hours and then treated with a fresh mixture of equal volumes of 4.8 per cent chromic acid and 3.6 per cent potassium bichromate solutions for 15–20 hours. After fixation, both pieces were washed thoroughly in running water, then stained according to Feulgen's method and turned into permanent squash preparations. Those which showed even a slight indication of pathologic degenerations were excluded from the present study.

In general, the first method was superior in the staining, but somewhat inferior in the spreading of the metaphase chromosomes, to the second method, although both methods had given very satisfactory results for most of the specimens. When two pieces of testes were taken, the two methods were always used; when only one piece was available, only the second method was used, unless the specimen had to be fixed in a shorter time. The two methods were used simultaneously as much as possible, in order to avoid errors due to artifacts which might be produced by either method.

Observations.—In each specimen metaphases of the spermatogonia and first spermatocytes were selected in which the chromosomes were spread out perfectly or nearly so as to cause no possible errors in the counting or analyses of their structures. The number of such first metaphases selected for observations in each specimen was at least fifteen and in some as many as fifty or sixty. Spermatogonial metaphases which were accurately analyzable were much smaller in number in every specimen, usually three or four. However, in each specimen the chromosome number was found to be exactly the same in all such spermatogonia, and the number found in the spermatogonia was also in complete agreement with the number determined from first metaphases.

While the first ten specimens were being studied, it was found that the chromosome number was definitely 48 in all but one. This exceptional one appeared to have less than 48 chromosomes. Those which were definitely found to have 48 chromosomes were analyzed as to relative sizes and positions of the kinetochores of all first-metaphase chromosomes, and the results were summarized in the form of an idiogram.[4]

Meanwhile, seven new testes were added to the 48-chromosome group, but at the same time three specimens were obtained in which the chromosome number was again found to be less than 48. These three and the similar one found previously were studied, and in every specimen the chromosome number was determined to be definitely 46 in the spermatogonia and 23 (bivalents) in the first spermatocytes. Thus it became known to the author that two different chromosomal types exist in Japanese, one with the diploid number of 48 and the other with that of 46.

The typical first metaphase of the 48-chromosome type is shown in Figure 1. Twenty-four bivalents, including the heteromorphic XY pair (*arrows*), are demonstrable in all figures, especially well in *A*.

While usually 24 bivalents like those shown here were observed at first metaphase, sometimes two univalents were found in place of a certain bivalent. In some of these exceptional metaphases, the univalents were found to be of equal size and to belong to a pair corresponding to one of the very small bivalents which was missing from the complement. It was not possible to tell, however, which was the one missing, since several of the very small bivalents were very much alike in size and shape. This type of exceptional metaphase was found in about 9 per cent of the metaphases observed. One example is shown in Figure 2, *A*.

In other exceptional metaphases the two univalents were different in size; one was very small, while the other was large and also peculiar in shape. These unequal univalents are shown in Figure 2, *B*, together with 23 bivalents. In such metaphases the heteromorphic chromosome pair was never observed. This left no doubt that the heteromorphic chromosome pair which was unquestionably the sex-determining element was a bivalent composed of two elements corresponding to the unequal univalents. These univalents undoubtedly represent X and Y chromosomes and thus provide another cytologic proof for the existence of a Y chromosome in the human male.

In the heteromorphic bivalent the Y chromosome is represented by the small terminal knob (see Fig. 1 and Fig. 2, *A*). It usually appears to be the fourth in size from the smallest chromosome of the haploid set, although its size relative to other chromosomes at the first metaphase may vary slightly. It is placed, therefore, at the twenty-second position in the author's idiogram for the human male. This is in contrast to the claims of other authors [1, 5] based on observations of mitotic metaphases that it is the smallest of the 48 chromosomes.

It is to be emphasized here that, with the exception of the XY and one autosomal pair described above, every first-metaphase chromosome was found to be never other than bivalent and always symmetrical in shape in all sixteen testes of the 48-chromosome type which were included in the present study.

The typical first metaphase of the 46-chromosome type is shown in Figure 3. Here the heteromorphic XY bivalent (*arrows*) similar in shape to the ones observed in the previous group and 22 instead of 23 autosomal bivalents are present. The total of 23 bivalents was observed regularly, except in some metaphases were X and Y chromosome were separate as two unequal univalents. Autosomal univalents like the ones found in the 48-chromosome group were not encountered in any of the four testes of this group. Whether this is a characteristic feature of this type or occurred only by chance is being investigated now by means of a larger number of first metaphases in this group. If the absence of the autosomal univalents is found to be constant, it might mean that the pair of chromosomes which appears as either a bivalent or univalents in the 48-chromosome type is entirely missing from this type.

In an attempt to find the reason for the difference between the 46- and 48-chromosome types, the first-metaphase chromosomes in the two types were compared individually according to their sizes and the relative lengths of the two arms.

The XY pair and about twenty large autosomes could be matched satisfactorily, but several of the very small ones were impossible to match with certainty because of the similarities of their sizes and shapes. It was suspected, however, that a certain very small pair in the 48-chromosome type was entirely absent in the other.

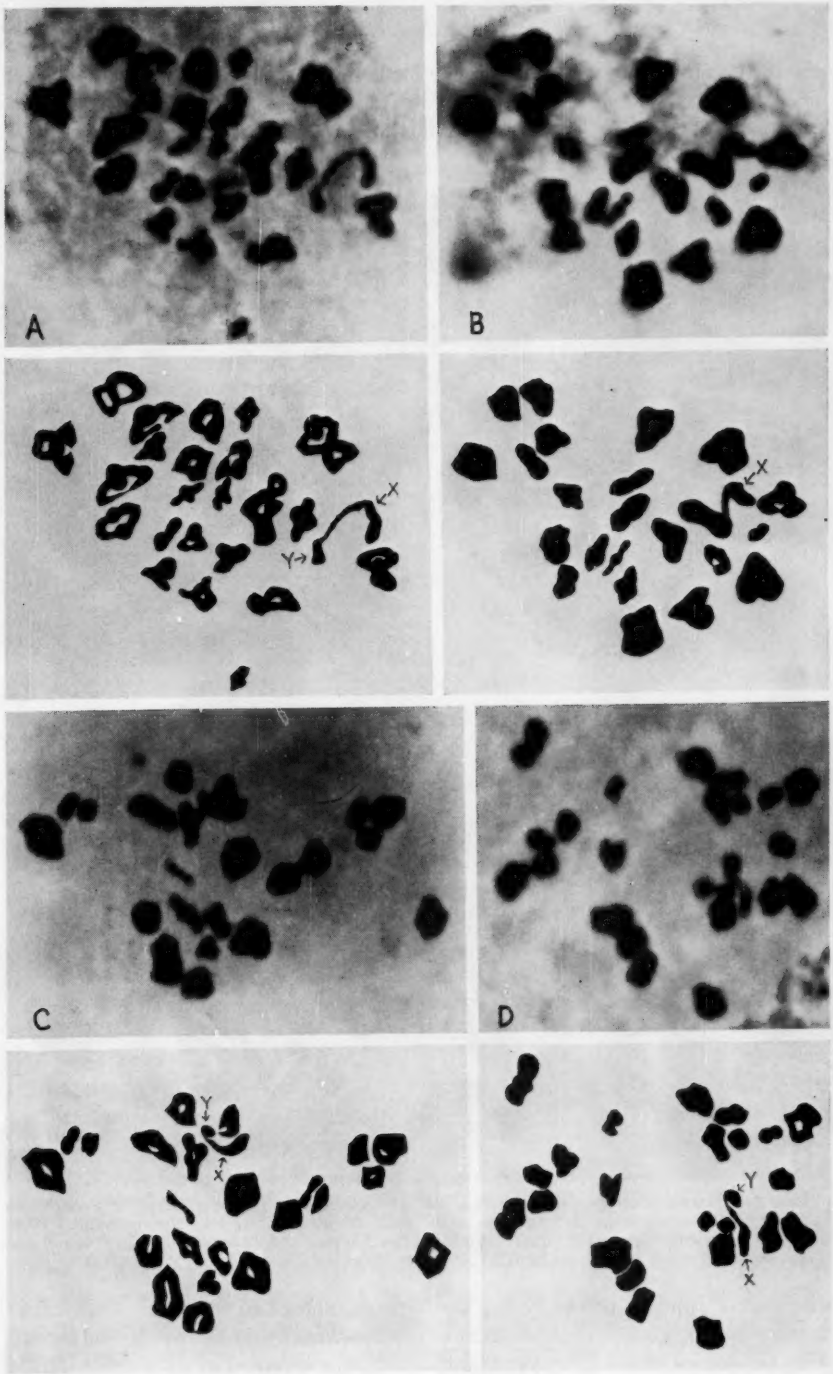
It was found further that in neither type had tri- or quadrivalent association of the chromosomes ever been observed at first metaphase or earlier stages. Nor had there been any indications of the pairing of any of the very small chromosomes with any other members except their homologues. This seems to indicate that the chromosome which is present in a pair in the 48-chromosome type and suspected to be absent entirely in the other type has no part homologous to any other chromosome. For this reason it may be regarded as a supernumerary chromosome.

Several of the very small autosomal pairs in the 48-chromosome type were checked for meiotic behavior which might identify the suspected supernumerary. In the resting nuclei a large condensed mass of chromatin was observed quite regularly. This mass, which is presumably the sex chromatin and is much too large to represent any of the small chromosomes under observation, appeared as a large heteropycnotic segment of a certain chromosome at early prophase. This chromosome will be specially dealt with in a later report. Other than this, no chromosomes were found to be extensively heteropycnotic during the resting or prophase stages. Thus the observation of meiotic behavior failed to identify a suspected supernumerary.

Since there is no reason to assume that this chromosome has lethal effects when present singly in addition to the diploid set of 46 chromosomes, and the two chromosomal types (46 and 48) found in the males presumably exist also in the females, some Japanese are expected to have 47 chromosomes resulting from the union of the sex cells with 24 chromosomes and those with 23. The males of this type, if found, would show 47 chromosomes in the spermatogonia and 23 bivalents plus a univalent supernumerary at first metaphase. A testis suspected to be of this type was obtained recently. The chromosome number in the spermatogonia was found clearly to be 47. The first metaphase is being studied now to see whether a univalent is present in addition to 23 bivalents. If this should turn out to be the case, it would become evident that a supernumerary is involved in the variation of the diploid chromosome number, and it would probably be identified exactly. Results of the studies of this specimen will be reported in a future communication.

Discussion.—Painter[1] was the first to report the existence of 48 chromosomes in the spermatogonia and 24 in the first spermatocytes of Negroes and whites. Several other authors[2] subsequently reported the same number in the spermatogonia of whites and Japanese. Forty-eight chromosomes were also demonstrated in embryonic tissues by some authors (e.g., Hsu[5]). On the other hand, deWiniwarter and Oguma[6] reported, in Belgian and Japanese, 47 chromosomes in the spermatogonia, 24 in the first spermatocytes, and 23 or 24 in the second spermatocytes. They believed that 47 was the correct diploid number and that the odd number was

FIG. 1.—Photomicrographs and camera lucida drawings of first spermatocyte metaphases of man with 48 spermatogonial chromosomes. Note in each figure the sex chromosome pair (*arrow*) and 23 autosomal bivalents. In *B* the Y chromosome is hidden behind the large chromosome below the X indicated by the arrow. *A* and *B* are taken from the same testis and *C* and *D* from two different testes. × *ca.* 3,500.


A
B
Y
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C
D
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due to the absence of a Y chromosome in the human males. Cytologic evidence against the XO theory was given by Painter in a clear figure of a late first spermatocyte anaphase showing X and Y chromosomes separating from each other in the

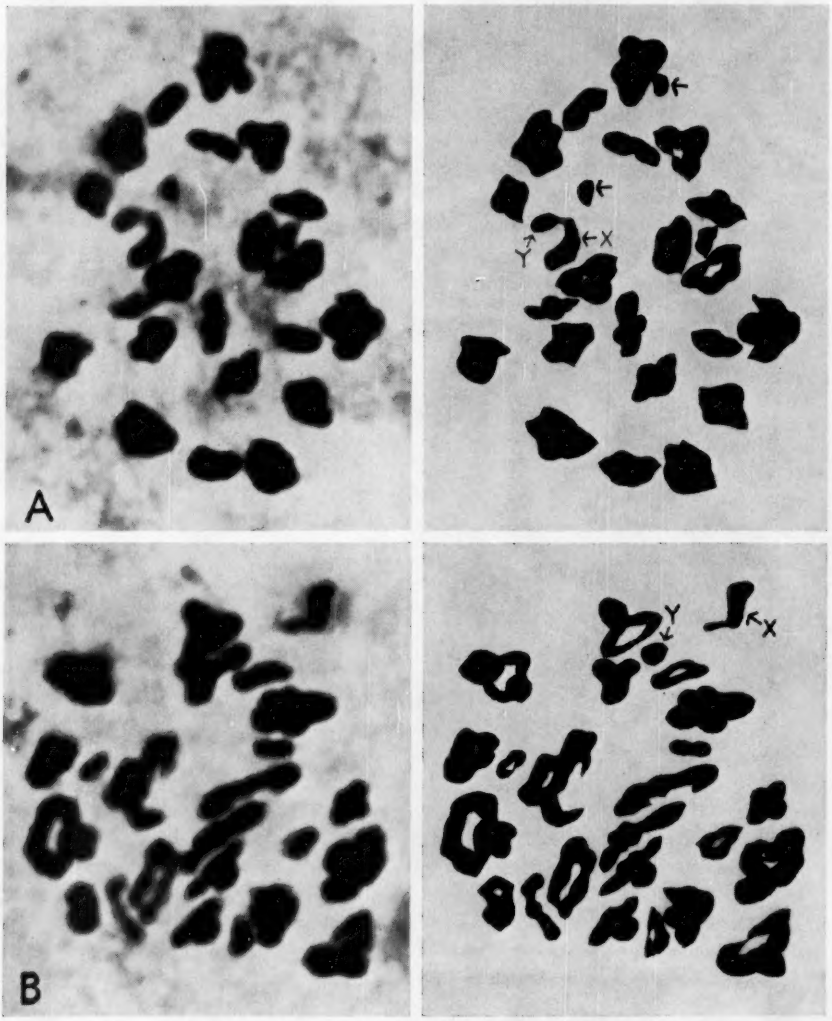

FIG. 2.—Photomicrographs and camera lucida drawings of first spermatocyte metaphases taken from the same testis in which spermatogonial counts indicated 48 chromosomes. Note in *A* two univalents (*arrows*) of equal size besides the XY pair and 22 bivalents. In *B* the X and Y are separate, while autosomes form 23 bivalents. For details see text. × *ca.* 3,500.

direction of opposite poles.[7] Since the Y chromosome had thus been clearly demonstrated cytologically, the findings of 47 chromosomes by deWiniwarter and Oguma were considered as not generally valid, and 48 was accepted as the correct diploid number of man.

Subsequently, as mentioned before, Tjio and Levan's[3] observations suggested that 46 was very possibly the correct diploid number of man, although they had no gonadal counts.[8]

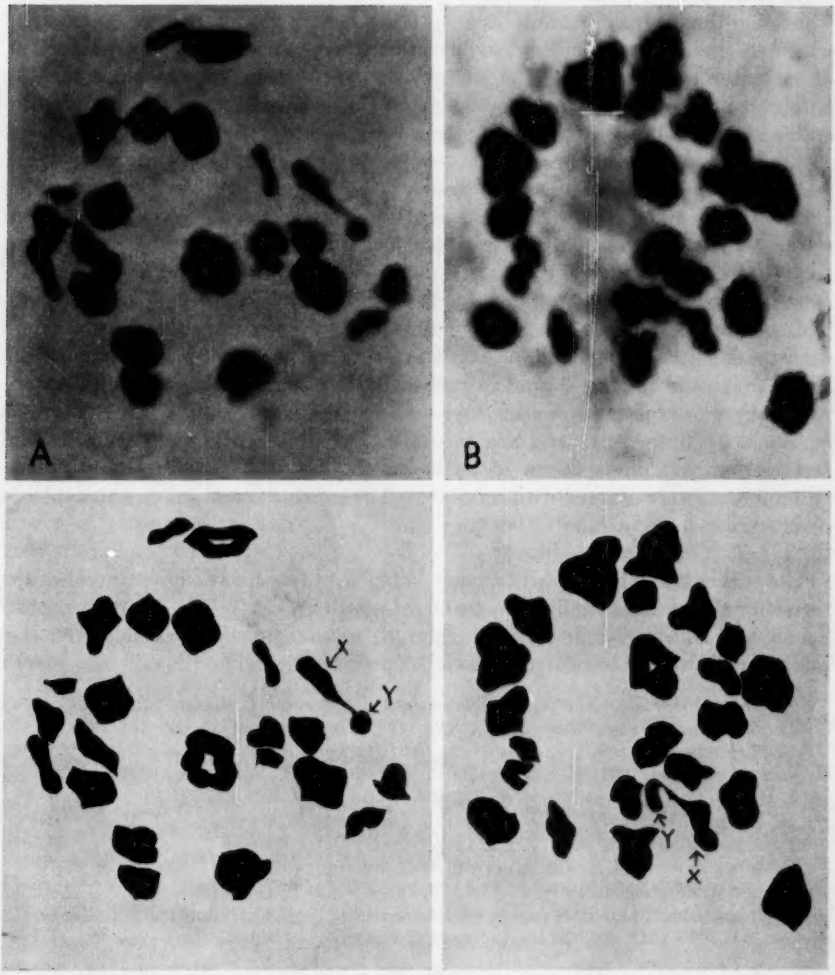

FIG. 3.—Photomicrographs and camera lucida drawings of first spermatocyte metaphases of different human testes in which 46 chromosomes are counted in the spermatogonia. Note the heteromorphic XY pair and 22 autosomal bivalents. × *ca.* 3,500.

Now that 46 chromosomes have been actually observed in the human germ cells, the same number found in the Swedish embryos could be regarded as representing their true diploid number. Moreover, since the present author has shown that 48 represents another type of diploid number in man, some of the testes and embryos, at any rate, in which 48 chromosomes have been demonstrated by other authors were probably of this type. Furthermore, the observation by the present

author of 47 chromosomes in one testis, which suggests the existence of a third type of diploid numbers in man, opens the possibility that the Belgian and Japanese materials used by deWiniwarter and Oguma were of this type.

All three diploid numbers found in Japanese thus seem to exist also in whites. The same three types probably exist in other human groups, such as Negroes, in which 48 chromosomes have already been demonstrated, although the proportion of the three types may be different from group to group. In Japanese the 48-chromosome type seems to be predominant, although the total number of men studied is too small as yet to indicate the exact proportion of the three types.

Summary.—The metaphase chromosomes of the spermatogonia and first spermatocytes were studied in twenty testes of Japanese. In sixteen of these the chromosome number was 48 in the spermatogonia and 24 (bivalents) in the first spermatocytes, whereas, in the remaining four, the number was 46 in the former tissue and 23 (bivalents) in the latter. Thus two diploid numbers were found to exist in man.

A comparison of the chromosomes of first metaphases of the 46- and 48-chromosome types suggests that a pair of simple supernumerary chromosomes of very small size is present in the latter type and absent in the former. This presumed supernumerary was expected to occur singly in some Japanese. They should have 47 chromosomes in the spermatogonia and one univalent in addition to 23 bivalents in first spermatocyte metaphases. A testis was obtained in which 47 spermatogonial chromosomes were counted with certainty. The constitution of the first-metaphase chromosomes is being investigated now.

The author is much indebted to many Japanese urologists who have provided the testes for the present studies and to Drs. Curt Stern, J. V. Neel, H. Plough, W. Schull, and D. MacDonald for much helpful criticism and advice as well as for the interest with which they have followed the present work.

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ON THE RELATIVE ABUNDANCE OF BIRD SPECIES

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One approach to the study of the structure of animal communities for which there is a considerable amount of raw data is the comparison of abundances of the species of a group of organisms in a given locality. The basic information is a list of species found and the abundance of each. This can best be plotted by ranking the species from commonest to rarest along the abscissa and plotting their abundances along the ordinate. For convenience, the abscissa is graduated logarithmically. Earlier investigations,[1] discussed elsewhere,[2] fit known statistical curves of uncertain biological meaning to the data. A more fruitful approach seems to be to predict curves on the basis of simple biological hypotheses and to compare these with the data. Three such hypotheses will be discussed here. First, however, the evidence cited by Lack,[3] combined with the observation that censuses yield similar results when taken in the same habitat either at the same time in different places or in the same place in different years, indicates, for birds at least, that the observed populations are not under the control of very local or rapid temporal processes; i.e., they are in a near-equilibrium state. The hypotheses will therefore be ones which yield an equilibrium or near-equilibrium population.

Hypothesis I: Nonoverlapping Niches.—The environment is compared with a stick of unit length on which $n - 1$ points are thrown at random. The stick is broken at these points, and the lengths of the n resulting segments are proportional to the abundances of the n species. This comparison is legitimate, since the mathematical part of the derivation can be used unchanged for spaces of higher dimension. The most complete discussion of the resulting distribution is given by Barton and David.[4] The expected length of the rth shortest interval is given by $(1/n) \sum_{i=1}^{r} [1/(n - i + 1)]$, so that the expected abundance of the rth rarest species among n species and m individuals is $(m/n) \sum_{i=1}^{r} [1/(n - i + 1)]$. This is plotted in Figure 1. Bird censuses from tropical forests[5] and many temperate regions fit this hypothesis almost perfectly. Quite frequently, however, the resulting curve is too steep; i.e., common species are too abundant, rare species too rare. These steep curves can be duplicated by considering the community as composed of two sticks of very different lengths (totaling unit length), each broken randomly into $n/2$ pieces. This is easily generalized to communities which are composed of several smaller ones, each obeying the original hypothesis. These composite communities are heterogeneous. The divergence from the ideal curve may, in fact, be regarded on this hypothesis as a measure of heterogeneity. Experimentally, for bird communities, this appears to explain most of the "steep" curves. For example, the census of Quaker Run Valley in Pennsylvania[6] yields a curve which is much too steep. When the censuses of the small pieces of the valley are plotted separately, they are in good agreement with the hypothesis. Some other populations of species, as of trees, do not seem to have this composite property and may require a different explanation.

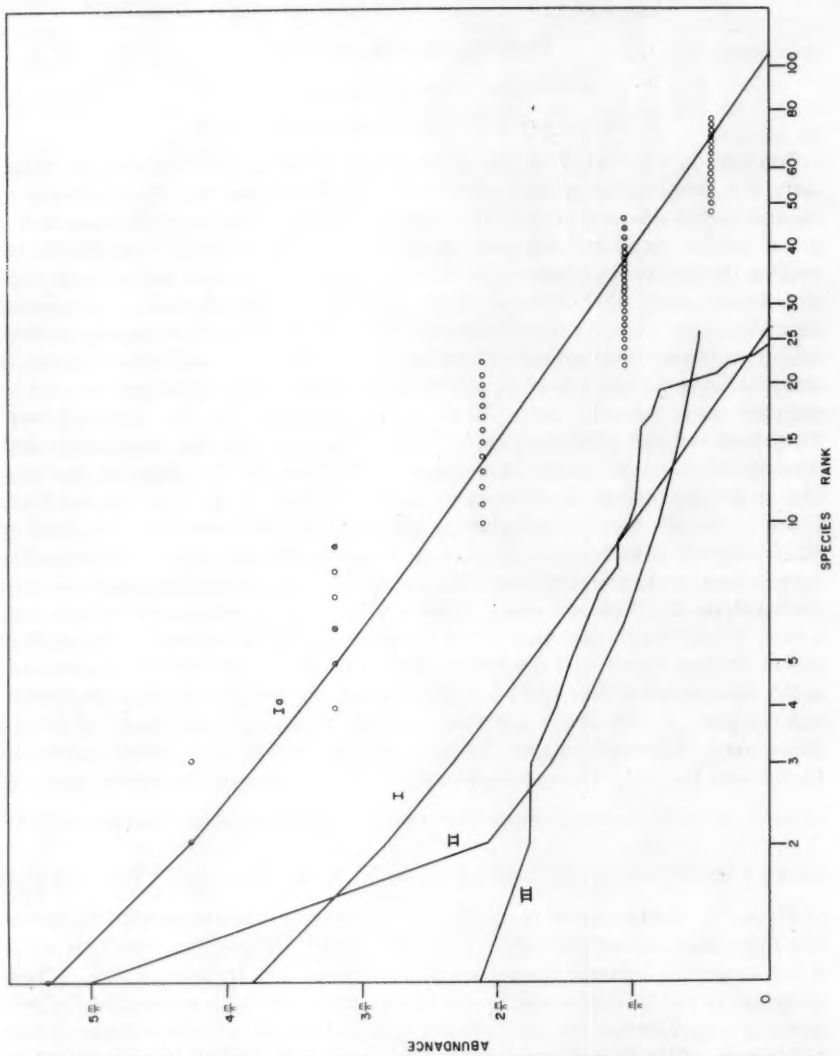

FIG. 1.—Comparison of the hypotheses. Curve *I* is the curve, on Hypothesis I, for 25 species, and curve *Ia* that, on the same hypothesis, for comparison with a census of 106 species. Curve *II* represents Hypothesis II for 25 species. Curve *III* is the mean of three sets of random tosses of 76 particles into 25 categories, illustrating Hypothesis III. The total number of individuals, is m; the total number of species, n.

Hypothesis II: Overlapping Niches.—The environment is again compared with a stick, but each species is now independent of the others. In other words, the abundance of any species is determined by the distance between a pair of points thrown at random onto the stick, n such pairs being thrown and the distances ranked and plotted as before. The probability density that the two points are

separated by distance x is given[7] by $2 - 2x$, and, by a theorem of Mood,[8] the expected abundance of the rth rarest species is obtained by breaking the area under the curve into n equal parts by vertical lines and taking the rth shortest length along the abscissa of the resulting segments. The result is $(\sqrt{n - r} - \sqrt{n - r - 1})/\sqrt{n}$. This is plotted in Figure 1 and is seen to predict commoner common species, commoner rare species, and scarcer species of intermediate abundance than does Hypothesis I. Since rare species appear never to be commoner than is predicted by Hypothesis I, the second hypothesis seems false. Higher-dimensional models have poorer fit. This hypothesis may have some truth for species in an environment with superabundant resources.

Hypothesis III: Niches Particulate, Not Continuous.—The abundance-determining factor is accumulated as independent discrete units by the various species. That is, the species can be compared with urns into which particles (units of "abundance") are tossed on independent random throws, each urn having equal probability. This hypothesis involves an extra independent variable, namely, the number of "tosses." When this number of tosses becomes infinite, by the law of large numbers, all species become equally abundant, since the number of individuals is bounded. When plotted by the Monte Carlo method for smaller numbers of tosses, the curve seems always to drop off at the right more suddenly than that of Hypothesis I, although the left end may vary in shape. While it may be possible to adjust the variables here to fit observed census data, the predictions based upon a priori biological postulates seem to fail. Furthermore, the variance seems much greater than that observed.

Conclusion: Hypothesis I—nonoverlapping, continuous niches—is much closer to observations than the other hypotheses. Since it has no parameters other than number of individuals and number of species, it is a much simpler hypothesis than previous ones, at least for "homogeneous" communities. The precise nature of the heterogeneity giving rise to the steeper curves sometimes observed seems obscure, but it can apparently be eliminated by breaking the community into small components. The failure of Hypotheses II and III suggests that, at least as a rough approximation, niches do not overlap much and are more continuous than discrete.

The author wishes to thank G. E. Hutchinson, who suggested the problem several years ago and who has encouraged continued interest in it. A fuller discussion will be given in a later paper.

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